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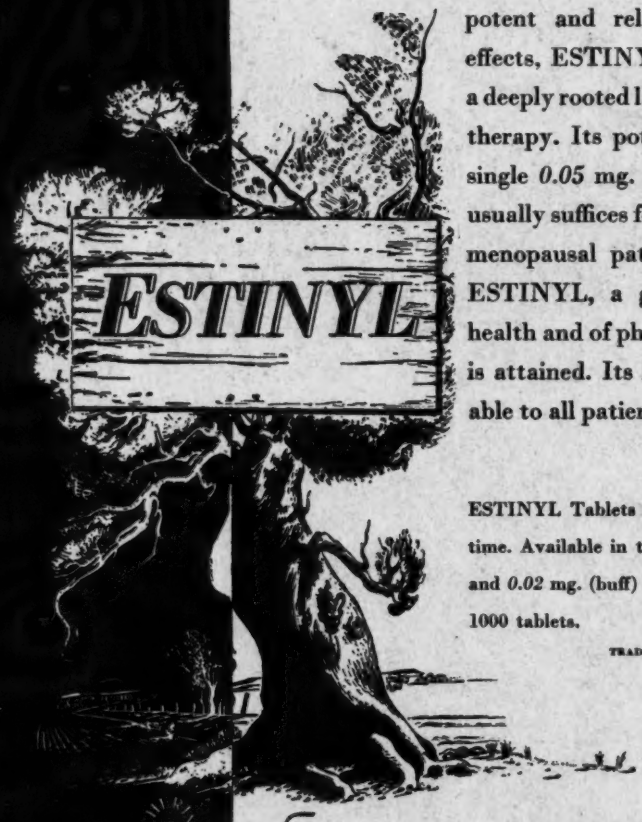

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ENDOCRINOLOGY

VOLUME 38 JANUARY, 1946 NUMBER 1

THE EFFECT OF PITUITARY ADRENOTROPHIC HORMONE ON THE CHOLESTEROL AND ASCORBIC ACID CONTENT OF THE ADRENAL OF THE RAT AND THE GUINEA PIG[1]

GEORGE SAYERS,[2] MARION A. SAYERS,[2] TSAN-YING LIANG AND C. N. H. LONG

From the Department of Physiological Chemistry, Yale University School of Medicine

NEW HAVEN

IT HAS ALREADY been reported that in the rat the adrenal cholesterol, a possible precursor of the adrenal cortical steroids, is lowered following administration of pituitary adrenotrophic hormone (Sayers, Sayers, Fry, White and Long, 1944). Preliminary studies (Sayers, Sayers, Lewis and Long, 1944) have indicated that the ascorbic acid content of this gland is also depleted by adrenotrophic hormone. This paper is a more complete report of these effects in the rat and extends the observations to the guinea pig, an animal which cannot synthesize ascorbic acid.

TECHNIQUE

The rats used were 24-day-old males of the Yale strain obtained from the Biochemical Laboratory of the Connecticut Agricultural Experiment Station through the courtesy of Dr. Rebecca Hubbell. Male guinea pigs weighing 300 to 450 grams were obtained from a dealer and maintained for a preliminary period on a stock diet[3] to which green stuffs were added. For a period of 5 days prior to administration of adrenotrophic hormone all green stuffs

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[1] Aided by a grant from the Committee for Research on Endocrinology, National Research Council, and the Fluid Research Fund, School of Medicine, Yale University.

[2] Present address: Department of Pharmacology, School of Medicine, University of Utah, Salt Lake City, Utah.

[3] Calf meal 91 per cent, cod liver oil 3 per cent, wheat germ 3 per cent and dried brewers' yeast 3 per cent.

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were withdrawn, and ascorbic acid (5 mg. per 100 gm. of body weight) was injected subcutaneously once per day. Only animals that grew well in the preliminary period were used.

Adenotrophic hormone (A.C.T.) was prepared[4] according to the method of Sayers, White and Long (1943). The hormone was injected intraperitoneally as an aqueous solution (pH 7.0) in a volume of 0.5 ml. in the case of the rats and in a volume of 1.0 ml. in the case of the guinea pigs. The dose of hormone was equal to 4 mg. per 100 gm. of body weight in both species.

At the end of the experimental period the animals were anesthetized with nembutal and exsanguinated by opening the inferior vena cava. The adrenals were weighed to the nearest 0.1 mg. on a torsion balance, one gland being used for the determination of ascorbic acid, the other for the determination of cholesterol. In the case of the guinea pigs, two samples of liver, each weighing approximately 3 to 4 grams, were quickly weighed, dropped into KOH, and used for the determination of glycogen. The remainder of the liver was used for the determination of ascorbic acid.

The ascorbic acid content of the tissues was determined by the method of Bessey (1938), and the cholesterol content by the method of Schoenheimer and Sperry as described by Sperry (1938).

RESULTS

1. *Effect of A.C.T. on the cholesterol and ascorbic acid content of the rat adrenal.*

(a) *Single dose of A.C.T.* The changes in the concentration of cholesterol in the adrenal of the rat following administration of a single dose of A.C.T. have been discussed in a previous paper (Sayers, Sayers, Fry, White and Long, 1944). The present data (Table I) extend these results to include intervals of 12 and 18 hours after hormone injection. At the end of three hours following the administration of A.C.T. the adrenal cholesterol has fallen to 50 per cent of the normal level and then slowly rises over the next 24-hour period to regain its original concentration in the gland.

TABLE I. EFFECT OF A SINGLE INJECTION OF A.C.T. ON THE CHOLESTEROL AND THE ASCORBIC ACID CONTENT OF THE ADRENALS OF 24-DAY-OLD RATS. FIGURES REPRESENT AVERAGES AND STANDARD ERRORS

Interval between administration of A.C.T. and removal of adrenals	Adrenal weight		Adrenal cholesterol			Adrenal ascorbic acid		
	no. rats	mg. per 100 gm. body wt.	no. rats	mg. per 100 mg. fresh tissue	mg. per 100 gm. body wt.	no. rats	mg. per 100 gm. fresh tissue	micrograms per 100 gm. body wt.
Controls	45	29.5±0.8	45	3.03±0.08	0.90±0.08	8	314± 6.1	89.3±5.5
20 Minutes	—	—	—	—	—	5	213± 6.1	63.9±4.7
1 Hour	6	30.0±0.8	6	2.62±0.24	0.79±0.09	5	134± 6.9	39.2±2.7
3 Hours	27	33.1±0.8	27	1.57±0.07	0.51±0.02	5	141±11.3	38.4±4.4
6 Hours	8	33.6±1.5	8	1.71±0.08	0.57±0.03	5	237±15.2	70.5±3.7
9 Hours	8	33.9±2.1	8	1.80±0.11	0.61±0.05	5	294± 6.1	88.7±2.1
12 Hours	4	30.7±0.9	4	1.98±0.24	0.60±0.06	4	328±16.6	100.0±5.6
18 Hours	5	34.0±2.6	5	2.25±0.12	0.77±0.07	5	338±12.5	112.6±7.3
24 Hours	9	35.9±1.1	9	2.89±0.10	1.05±0.06	4	374± 4.8	127.8±5.6

Following injection of A.C.T., the ascorbic acid content of the

[4] The authors are indebted to Dr. J. B. Fishman for the preparation of the hormone used in this study.

adrenal decreases more rapidly than that of cholesterol. Within twenty minutes after the administration of the hormone, it has fallen to two-thirds of its initial value, and in 1 hour reaches a minimum level of less than one half its initial concentration. Between 1 and 3 hours the rate of disappearance of ascorbic acid from the gland apparently equals the rate of its reaccumulation, since no essential changes occur during this period. However, from this point on, the ascorbic acid rapidly increases in concentration, so that the initial level is regained between 9 and 12 hours after the injection. Beyond 12 hours ascorbic acid continues to accumulate in the gland, so that

TABLE II. EFFECT OF PROLONGED ADMINISTRATION OF A.C.T.* ON THE CHOLESTEROL AND ASCORBIC ACID CONTENT OF THE ADRENALS OF 24-DAY-OLD RATS. FIGURES REPRESENT AVERAGES AND STANDARD ERRORS

Interval between last injection of A.C.T. and removal of adrenals	No. rats	Adrenal wt. mg. per 100 gm. body wt.	Adrenal cholesterol		Adrenal ascorbic acid	
			mg. per 100 mg. fresh tissue	mg. per 100 gm. body wt.	mg. per 100 gm. fresh tissue	micrograms per 100 gm. body wt.
Controls	45	29.5 ± 0.8	3.03 ± 0.08	0.90 ± 0.08	314 ± 6.1†	89.3 ± 5.5†
3 Hours	8	45.0 ± 1.7	1.23 ± 0.07	0.56 ± 0.04	195 ± 12.6	88.8 ± 8.1
16 Hours	3	47.8 ± 1.5	3.23 ± 0.26	1.53 ± 0.08	293 ± 33.7	139 ± 11.3
38 Hours	4	47.2 ± 4.8	5.06 ± 0.21	2.39 ± 0.27	426 ± 12.3	202 ± 20.8
74 Hours	4	41.7 ± 1.8	5.03 ± 0.23	2.10 ± 0.16	414 ± 28.8	173 ± 12.4

* Injected thrice daily for 3 days, total dose 20 mg. per 100 gm. body wt.
† Average of 8 rats.

amounts greater than normal are found 24 hours after the injection of a single dose of A.C.T.

(b) *Prolonged administration of A.C.T.* In a previous study (Sayers, Sayers, Fry, White and Long, 1944), it was noted that the injection of A.C.T. thrice daily for three days into 24-day-old rats (total dose, 2–5 mgms.) not only produced marked hypertrophy of the adrenals, but also, in the case of the larger dose, actually increased the concentration of adrenal cholesterol by some 40 per cent. Although the adrenals were in all cases removed some 16 hours after injection, in no instance was the adrenal cholesterol found to be lower than in uninjected animals.

Since the question might be raised that a lowering of adrenal cholesterol was not invoked after continued intermittent injection of A.C.T., experiments were carried out in which the ascorbic acid, as well as the cholesterol content of the adrenals, was determined at various intervals after the last dose of a series of injections of A.C.T. The results are shown in Table II.

It will be observed that the response to the last of a series of injections of A.C.T. is as great, if not greater, than that invoked by a single injection. It is apparent from the values observed 38 hours after the next to the last injection, when the immediate effects of A.C.T. must have subsided, that not only were the glands 60 per

cent larger than normal, but their cholesterol content was some 66 per cent greater, and their ascorbic acid content 36 per cent greater. However, 3 hours after the last injection of A.C.T., the same profound fall in the levels of these constituents is observed as after a single dose.

2. *Effect of A.C.T. on the cholesterol and ascorbic acid content of the guinea pig adrenal.*

In considering the effect of A.C.T. on the guinea pig adrenal, account must be taken of the fact that withdrawal of ascorbic acid for a period of 48 hours in itself causes a 30 per cent decrease in adrenal ascorbic acid and a slight fall in adrenal cholesterol. There is also a considerable loss of ascorbic acid from the liver.

In order to avoid the complication introduced by varying periods since the injection of the last dose of ascorbic acid, all the guinea pigs cited in Table III were sacrificed after a fasting period of 24 hours and at a time which was 36 hours after the last injection of ascorbic acid The A.C.T. injection was, therefore, given at different

TABLE III. EFFECT OF A SINGLE INJECTION OF A.C.T. ON THE CHOLESTEROL AND ASCORBIC ACID CONTENT OF THE ADRENALS OF GUINEA PIGS. ADRENALS REMOVED AT END OF A 24 HOUR FAST AND 36 HOURS AFTER LAST INJECTION OF ASCORBIC ACID. FIGURES REPRESENT AVERAGES AND STANDARD ERRORS

Interval between administration of A.C.T. and removal of adrenals	No. guinea pigs	Adrenal weight mg. per 100 gm. body wt.	Adrenal cholesterol		Adrenal ascorbic acid		Liver ascorbic acid
			mg. per 100 mg. fresh tissue	mg. per 100 gm. body wt.	mg. per 100 gm. fresh tissue	micrograms per 100 gm. body wt.	mg. per 100 gm. fresh tissue
Controls	9	44.6±1.9	4.51±0.22	2.03±0.15	137±7.9	61.7±5.4	13.5±0.95
3 Hours	6	47.3±3.3	3.53±0.17	1.67±0.15	75±4.4	35.1±1.9	—
6 Hours	5	44.1±3.5	3.78±0.13	1.66±0.11	66±3.9	29.2±2.5	14.1±1.34
12 Hours	6	52.1±0.7	3.08±0.23	1.61±0.13	65±5.8	33.8±3.1	14.7±1.56
18 Hours	6	51.0±5.3	3.11±0.22	1.60±0.23	86±8.7	43.4±4.8	—
24 Hours	6	57.5±3.1	3.98±0.28	2.32±0.26	119±2.4	67.8±3.6	13.4±0.37
12 Hours after gelatin	6	44.8±2.6	4.61±0.44	2.17±0.20	125±8.4	55.1±4.4	15.1±1.51

times before the end of the 24-hour fast. The changes observed are, therefore, solely due to the influence of the hormone. As a further precaution, all the animals were kept in a constant temperature room (84 ± 1°F.) for a week before and during the experiment.

The change in adrenal cholesterol in the guinea pig (Table III) following the administration of a single dose of A.C.T. shows the same pattern as in the case of the rat. However, there is a greater percentage fall in the rat, although if the results are expressed as mg. of cholesterol per 100 gm. of body weight, the loss is the same in both species. Thus, in the guinea pig 3 hours after injection of A.C.T., the decrease amounted to 0.37 mg. per 100 gm. of body weight, while in the rat at the same period, it was 0.39 mg. per 100 gm. of body weight.

As might be expected, the changes in adrenal ascorbic acid in the guinea pig were quite different from those in the rat. Following administration of A.C.T. to the guinea pig, while there was the same marked depletion of the vitamin from the gland, the rapid return to

almost normal levels did not occur. However, if a sufficient period of recovery is allowed, a slow reaccumulation of adrenal ascorbic acid is evident. Thus, 18 hours following A.C.T. administration there was some return of ascorbic acid to the gland, and at 24 hours an appreciable amount had again been deposited. It would appear that where abundant stores of the vitamin are present in the tissues, the levels in the adrenal can be replenished by its withdrawal from the blood.

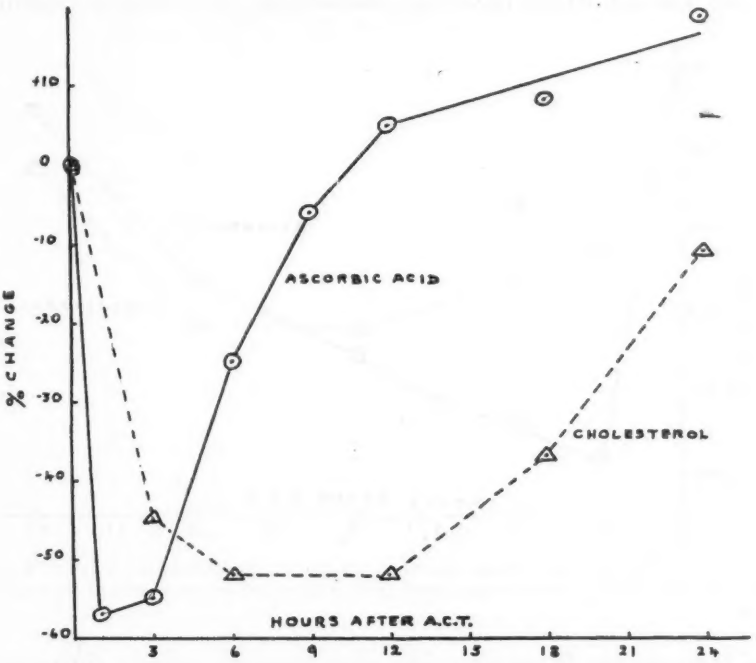

FIG. 1. The effect of a single injection of adrenotrophic hormone (A.C.T.) (4 mg. per 100 gms. body weight) on the adrenal cholesterol and ascorbic acid of the rat.

The loss of adrenal ascorbic acid expressed in terms of unit weight of gland or of body weight were greater in the rat than the guinea pig.

Figures 1 and 2 illustrate the differences in the response of the two species to A.C.T.

The specificity of the effect of A.C.T. on the adrenal is shown by the fact that the concentration of ascorbic acid in the liver of the guinea pig was not affected by A.C.T. (Table III), a finding that is in keeping with numerous observations on various tissues of the rat.

The changes in the adrenal are not due to some non-specific effect

of protein injection, since animals sacrificed 12 hours following intraperitoneal injection of gelatin (in an amount equivalent to amount of A.C.T. injected) had the same levels of adrenal ascrobic acid and cholesterol as the controls (Table III).

The above analyses were made on the whole adrenal, cortex plus medulla. A few analyses were made on cortical tissue alone. In two control animals the following values were obtained, adrenal cholesterol 6.70 and 4.58 mg. per 100 gm. of fresh tissue, and adrenal ascorbic acid, 140 and 104 mg. per 100 gm. fresh tissue. Six hours after injection

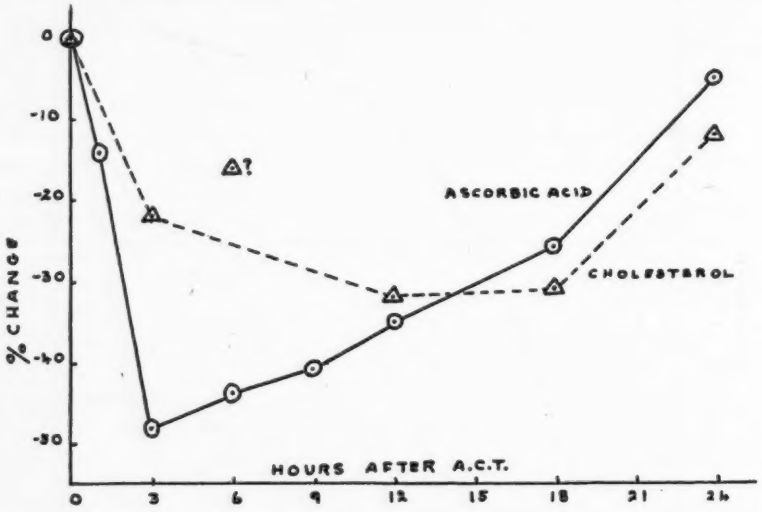

FIG. 2. The effect of a single injection of adrenotrophic hormone (A.C.T.) (4 mg. per 100 gms. body weight) on the adrenal cholesterol and ascorbic acid of the guinea pig.

of hormone the following values were obtained, adrenal cholesterol 3.89 and 4.07 mg. per 100 gm. fresh tissue and adrenal ascorbic acid 55 and 64 mg. per 100 gm. of fresh tissue. These results indicate that the alterations observed to take place in the whole gland reflect changes in the cortex.

3. *Effect of a single dose of A.C.T. on liver glycogen of the guinea pig.*

The guinea pigs were injected with A.C.T. 24 hours after the beginning of fasting and sacrificed at various intervals thereafter. A sharp rise in the glycogen content of the liver took place 6 hours after hormone injection, which continued through 12 hours (Table IV). After 24 hours the level of glycogen was the same as that of controls fasted 48 hours.[5] Glycogen deposition following the administra-

[5] This rise in the glycogen content of liver on extension of fasting is a well established phenomenon.

tion of the hormone appears to be greatest at the time when the cholesterol content of the gland is at a minimum. Following the return of cholesterol to pre-injection levels, the glycogen content of the liver also returns to normal. This agrees with previous findings in the rat (Sayers, Sayers, Fry, White and Long, 1944). The evidence, although indirect, suggests that the depletion of cholesterol in the adrenal is associated with an increased secretion of those steroids

TABLE IV. EFFECT OF A SINGLE INJECTION OF A.C.T. ON THE GLYCOGEN OF THE LIVER OF GUINEA PIGS. FIGURES REPRESENT AVERAGES AND STANDARD ERRORS

Interval between administration of A.C.T. and removal of liver	Period of fasting hours	Number guinea pigs	Liver glycogen gm. per 100 gm. fresh tissue
Controls	24	5	0.038 ±0.007
6 Hours	30	5	0.38 ±0.12
12 Hours	36	6	0.74 ±0.13
24 Hours	48	6	0.37 ±0.06
Controls	48	7	0.37 ±0.12

of the type of corticosterone that are known to increase the liver glycogen.

4. *Effect of administration of ascorbic acid on the cholesterol concentration of the rat adrenal.*

Sodium ascorbate (30 mg. per 100 gm. body weight) was injected subcutaneously into 24-day-old rats at intervals (1 hour and 3½ hours) following the administration of A.C.T., in order to determine whether increasing the level of ascorbic acid in the adrenal during the early stages of depletion would influence the return of cholesterol into the adrenal. The administration of ascorbic acid resulted in appreciably higher levels of this substance in the adrenals of the treated animals. However, animals treated with ascorbic acid and autopsied at 3, 6 and 9 hours after A.C.T. injection showed the same levels of cholesterol in the adrenals as animals administered A.C.T. alone.

DISCUSSION

It appears that A.C.T. has a common action in both the rat and the guinea pig, namely, depletion of the level of cholesterol and ascorbic acid in the adrenal. The difference between these two species is, however, shown in the rate at which ascorbic acid reaccumulates in the gland. During this period the guinea pig is limited in its ability to replenish the vitamin in the adrenal from the stores available in the blood and tissues, and in consequence, the restoration of normal levels in the gland is greatly retarded. On the other hand, the replenishment of the cholesterol content occurs at about the same rate in both species. The changes brought about by A.C.T. in the adrenal appear to be specific for this organ, since in the guinea pig and rat single

injections of A.C.T. do not decrease the cholesterol and ascorbic acid content of other tissues.

The depletion of ascorbic acid and cholesterol from the adrenal following administration of A.C.T., together with the increase in liver glycogen, suggests that these substances are concerned with the elaboration of the cortical hormones. This view is strengthened by the fact that A.C.T. does not decrease the cholesterol and ascorbic acid content of other tissues. The chemical relationships between cholesterol and the steroid hormones indicate that it may be a precursor of the cortical hormones. It is now apparent that cholesterol does undergo transformations of this character in the organism. Thus Bloch, Berg and Rittenberg (1943) observed that cholesterol tagged with deuterium was converted into cholic acid, while Bloch (1945) has found that similarly labeled cholesterol, when fed to women, was converted into pregnanediol, the excretory product of progesterone.

It is more difficult to assign so definite a role to the adrenal ascorbic acid. Although the dye method used in this study would only determine reduced ascorbic acid, a few experiments in which dehydroascorbic acid was also determined have shown that conversion of ascorbic acid to the oxidized form will not account for the quantities that disappear. It must, therefore, either have been converted into products undetectable by the methods employed or have actually left the gland.

In this last regard, Zwemer, Lowenstein and Pines (1940) have already suggested that the adrenal contains water soluble steroids in which the steroid nucleus is associated with ascorbic acid. In a private communication Lowenstein states that he has actually isolated such a compound that possesses the biological activity of the cortical hormone. If this important claim is substantiated, then the role of ascorbic acid in cortical function would be established. Such a fact would also explain the disparity in the rate of decline of adrenal ascorbic acid and cholesterol after stimulation of the gland by A.C.T. The more rapid fall in ascorbic acid may represent the release of the hormone itself, while the slower decline in cholesterol would occur as this material is converted into further quantities of hormone.

The apparent relationship between adrenal cortical function and ascorbic acid raises the question as to the degree of adrenal cortical impairment in scurvy. Although it has been reported by some investigators (Ratsimamanga, 1939, 1944) that the symptoms of scurvy may be alleviated and life prolonged by injections of cortical hormone, others hold that the two conditions are not related. The lack of resistance of the scorbutic guinea pig to various stresses, together with certain similarities between scurvy and adrenal cortical insufficiency, is at least worthy of further study, particularly in the light of the present findings.

It has already been pointed out that many types of stress deplete

the cholesterol content of the adrenal (Sayers, Sayers, White, Fry and Long, 1944) and that this is a consequence of the adrenal cortical stimulation following the secretion of adrenotrophic hormone. There is an equally large number that deplete the adrenal ascorbic acid, some of which have been reported in detail (Sayers, Sayers, Liang and Long, 1945. Torrance, 1940). No such changes occur in the adrenals of hypophysectomized animals exposed to similar conditions, and this fact, together with the results of the exogenous action of A.C.T., would seem to leave little doubt that the alterations observed in adrenal ascorbic acid and cholesterol are directly associated with the activation and increased secretory level of the adrenal cortex induced by the release of the adrenotrophic hormone from the anterior pituitary.

SUMMARY

The injection of highly purified adrenotrophic hormone (A.C.T.) into rats and guinea pigs is followed by a prompt fall in adrenal ascorbic acid and a slower fall in adrenal cholesterol. The levels of these substances in other tissues are not affected. Following such injections, the adrenal cholesterol slowly returns to the initial levels in 24 hours in both species. The rate of return of adrenal ascorbic acid is quite rapid in the rat. In the guinea pig the level remains depressed for some hours and then slowly rises, but 24 hours after the injection the level is still subnormal. Coincident with the fall in adrenal ascorbic acid and cholesterol there is an increased deposition of liver glycogen in both the rat and guinea pig. It is suggested that these changes in adrenal ascorbic acid and cholesterol under the influence of A.C.T. are associated with the formation and release of the adrenal cortical hormones.

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THE INFLUENCE OF DIETHYLSTILBESTROL ON ALLOXAN DIABETES PAIRED FEEDING EXPERIMENTS[1]

R. G. JANES AND H. DAWSON

From the Department of Anatomy, College of Medicine, State University of Iowa

TWO SOMEWHAT conflicting views appear in the literature concerning the action of estrogenic substances on diabetes. One view is that diethylstilbestrol acts as a diabetogenic agent and the other that estrogenic substances relieve diabetic symptoms to some degree. Ingle and Nezamis (1943) found that when rats were partially pancreatectomized and force-fed with a high carbohydrate diet, the administration of diethylstilbestrol caused the animals to become glycosuric. The glycosuria disappeared, however, following the withdrawal of this drug. On the other hand, Nelson and Overholser (1936) showed that the injection of estrone usually caused a reduction in urine and blood sugar values in monkeys which had been partially or totally depancreatized. In addition, diabetes in human beings seems to be somewhat ameliorated following estrogen therapy, as evidenced by lowering the requirements for insulin (Schöne, 1940. Spiegelman, 1940. Thaddea and Hampe, 1940).

Because of these somewhat conflicting observations, the present study has been made to determine what effect diethylstilbestrol might have on rats which were made diabetic with alloxan, studied under paired feeding conditions.

METHODS

Male rats of the Long-Evans strain were given intraperitoneal injections of 125 mg./kg. of alloxan. Within a few days after receiving this drug the diabetic rats were placed on the following diet which was fed throughout the experiment:

Casein	350 gm.
Sucrose	600 gm.
Corn Starch	600 gm.
Yeast (Pabst)	200 gm.
Salt Mixture*	50 gm.
Crisco	100 gm.
Wesson Oil	100 gm.
Choline	400 mg.
Haliver Oil	4 cc.

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* Hubbell, R. B., L. B. Mendel and A. J. Wakeman: *J. Nutrition* 14: 273. 1937.

The animals were observed during a preliminary period of four to five weeks while the course of the diabetes became stable. At weekly intervals during this period the weights of the animals and food intake were recorded; also, determinations were made on the blood for content of sugar and non-protein nitrogen (N.P.N.) and on the urine for content of sugar and ketone bodies. At the conclusion of this observation period, 24 rats of the group were paired according to similarity of weights and blood sugar values. By correlating these criteria, the degree of diabetes was approximately the same for both rats of a single pair, although it varied in severity among the different pairs.

For a period of 21 days, one rat from each of eight pairs was given daily subcutaneous injections of 100 μg of diethylstilbestrol dissolved in $\frac{1}{5}$ cc. of peanut oil. The mates of these animals, serving as controls, were given peanut oil only. Two pellets of diethylstilbestrol (totalling 18–20 mg.) were implanted in one animal from each of the four remaining pairs. Their controls received no treatment. Since the observations made on the animals which received pellets and those which were injected with diethylstilbestrol were very similar, the experimental data submitted is an average of the two groups. The rats which received diethylstilbestrol developed a mild anorexia and were allowed to feed ad libitum. Their food intake was weighed daily so that the control mate for each animal injected with diethylstilbestrol could be limited to this same amount of food during the following 24 hour period.

All determinations were made on non-fasting animals, except for the final tests which were made after the rats had been fasted for 24 hours. Blood sugar determinations were made by the Shaffer-Hartman-Somogyi micro-method and blood N.P.N. was determined by the method of Kock-McMeekin. Twenty-four hour urine specimens were preserved in a few drops of toluol. Urine sugar was determined by the Shaffer-Hartman-Somogyi macro-method. Qualitative estimations for acetone bodies were made by the sodium nitroprusside test. At the end of the 24 hour fast, preliminary to making final tests, blood was taken from the dorsal vein of the tail for N.P.N. and sugar studies. The rats were then anesthetized with sodium amytal. Approximately three grams of liver were removed ($1\frac{1}{2}$ grams from each lobe) and frozen quickly in a mixture of carbon dioxide and ether in preparation for the determination of liver glycogen levels by the method of Good, Kramer and Somogyi. The adrenals, pituitaries, left kidneys and testes were removed and weighed.

RESULTS

The diabetes of the individual pairs of rats was somewhat variable. Although the pairs which were severely diabetic did not receive insulin, they gained weight during the pretreatment period. The pretreatment averages of body weight and N.P.N. shown in Fig. 1 for the twelve pairs of rats represent the actual values of data obtained on that day, but data for food consumption, blood sugars, urine sugars and urine volumes are averages of two or three determinations made at weekly intervals. The criteria of the number of weekly determinations used to establish the pretreatment values were based

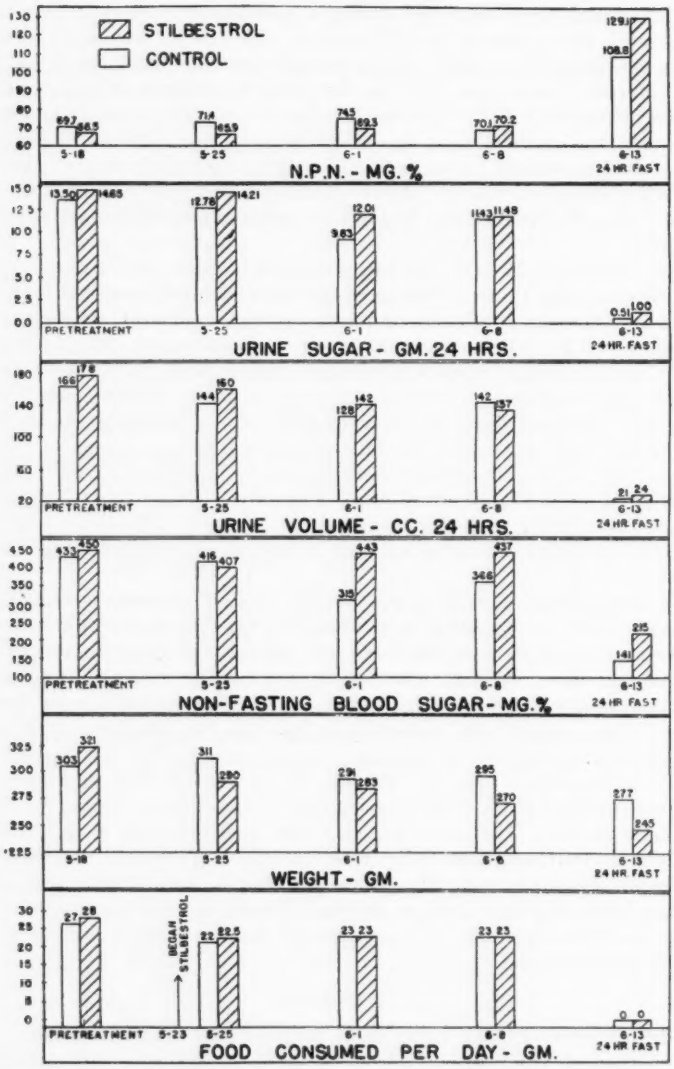


FIG. 1. Average values for 12 pairs of alloxan diabetic rats showing data for controls and animals treated with diethylstilbestrol during periods of paired feeding.

on the stability of the diabetes. Only those values were used which indicated that the diabetes was stable.

Food consumption was checked daily after the rats were paired on May 20th. There was some daily variation in the amount of food the stilbestrol-treated animals consumed and, therefore, in the amount

given to the controls. In order to eliminate some of the variation and to give a more correct value for food consumption for the 12 pairs of animals, tabulated averages beginning with May 25th data represent daily averages of five days' food consumption—two days before and two days after the date indicated. Within two days after diethylstilbestrol was given to the rats, food consumption dropped and continued at this rather constant lower level throughout the experiment.

When the animals were paired, those which were to receive diethylstilbestrol were slightly heavier than the controls (see Fig. 1). With reduced food intake all animals lost weight but this was more pronounced in animals receiving diethylstilbestrol than in the controls. This was particularly true after the 24 hour fast on June 13th.

The blood sugar values of the control and experimental rats were quite similar during the first two test periods. During the final periods, however, the average values for the control animals were decreased while those of the animals receiving diethylstilbestrol were maintained at approximately the pretreatment level. The fact that the control values were lower can be accounted for partly in that their daily allotment of food was given to them at one time and some of the animals consumed it before the end of the 24 hour period. Consequently, some of these animals were fasting by the time the blood sugars were determined. This discrepancy was partially corrected during the last week of the experiment by dividing the food allotment into two equal parts which were fed at 12 hour intervals. The volume of urine showed a rather steady decline starting on May 2nd in both control and treated rats. These lowered values correlate, in general, with the reduced food consumption. The decrease in urine volume correlates, also, with the reduction in urinary sugar values (see Fig. 1).

Blood N.P.N. values were somewhat variable in individual animals but when the averages of the twelve pairs of rats were considered the N.P.N. levels were quite similar for the controls and those animals treated with diethylstilbestrol. On fasting, the N.P.N. values were increased for both groups but the increase was particularly striking in the rats treated with diethylstilbestrol (see Fig. 1).

Data from two pairs of the diabetic rats, which were considered representative for the group of animals, are shown in Table 1. One pair was severely diabetic, the other had a mild diabetes. Diethylstilbestrol was given to one rat in each pair. In general, the values obtained for these selected animals follow a pattern similar to that of the averages for all rats which are shown in Fig. 1 but illustrate some of the variations in data which are obscured in the averages. Certain differences between these individual data and those of the averages in Fig. 1 are apparent. For instance, all values are higher for the animals which were severely diabetic, except for body weights and blood N.P.N. determinations. The non-fasting N.P.N. values were quite variable in all of the animals. Following the 24 hour fast, although they remained in the same range for the mild diabetics, the

TABLE 1. DATA FROM REPRESENTATIVE PAIRS OF DIABETIC RATS

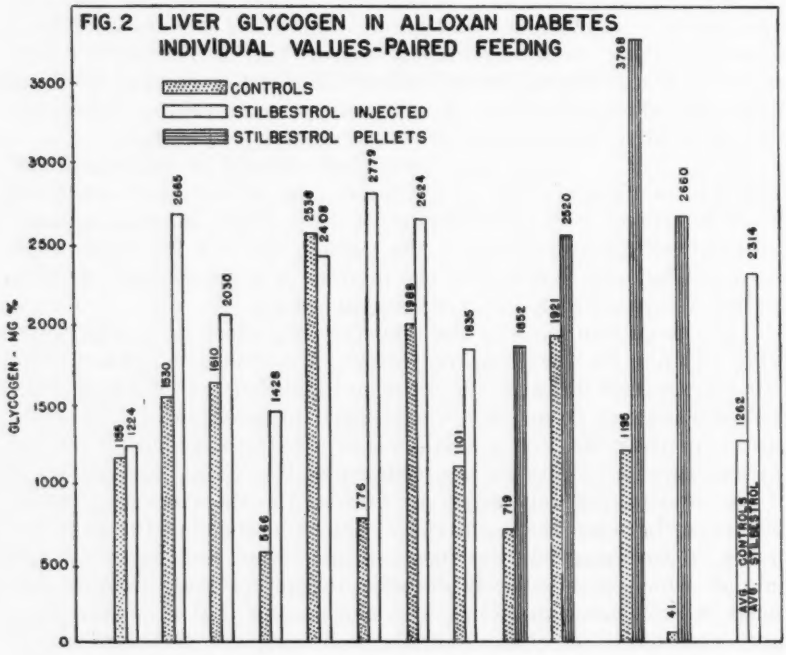
A. Severe diabetes

Control Animal #3							*Injected with stilbestrol* Animal #9						
Date	Wt. gm.	Food 24 hr. gm.	Blood Sugar mg. %	Urine 24 hr. cc.	Urine Sugar 24 hr. gm.	N.P.N. mg. %	Date	Wt. gm.	Food 24 hr. gm.	Blood Sugar mg. %	Urine 24 hr. cc.	Urine Sugar 24 hr. gm.	N.P.N. mg. %
							(Pretreatment):						
4–26	268	30	529	224	18.68	80	4–26	272	31	575	198	15.96	74
							5–1 Stilbestrol injections started						
5–2	263	27	556	230	17.36	80	5–2	286	27	487	176	15.33	65
5–9	262	20	392	192	14.71	88	5–9	255	24	525	126	9.95	57
5–16	252	24	321	174	13.26	77	5–16	238	24	535	130	11.78	52
5–21[1]	235	0	108	18	.20	124	5–21[1]	218	0	122	26	0.95	139

B. Mild diabetes

Control Animal #16							*Injected with stilbestrol* Animal #10						
Date	Wt. gm.	Food 24 hr. gm.	Blood Sugar mg. %	Urine 24 hr. cc.	Urine Sugar 24 hr. gm.	N.P.N mg. %	Date	Wt. gm.	Food 24 hr. gm.	Blood Sugar mg. %	Urine 24 hr. cc.	Urine Sugar 24 hr. gm.	N.P.N. mg. %
							(Pretreatment):						
4–26	313	20	342	40	4.81	66	4–26	330	19	275	65	4.58	62
							5–1 Stilbestrol injections started						
5–2	329	15	294	72	8.75	72	5–2	339	15	300	65	4.19	59
5–9	310	15	152	50	4.73	42	5–9	304	15	299	80	5.12	71
5–16	308	17	193	51	5.65	46	5–16	285	17	359	70	4.47	59
5.21[1]	295	0	79	12	0.237	59	5–21[1]	272	0	97	27	0.0	73

[1] Fast 24 hours.



N.P.N. values for the severely diabetic animals showed considerable elevation, particularly for animals given diethylstilbestrol.

The administration of diethylstilbestrol to the diabetic animals during paired feeding caused a marked elevation in liver glycogen (Fig. 2). This was true for all of the animals which received diethylstilbestrol with one exception. In this exception, the control animal had an extremely high value for liver glycogen. In general, implanted pellets were more effective in elevating liver glycogen than were injections of diethylstilbestrol. When values for animals which were injected with diethylstilbestrol and those which received pellets were averaged, their liver glycogen values showed an increase of 91% over

TABLE 2. ORGAN WEIGHTS OF DIABETIC ANIMALS TREATED WITH DIETHYLSTILBESTROL

(Mg./100 gm. body weight)

	Controls	Stilbestrol
Number of rats	12	12
Adrenals (2)	18.8	20.8
Kidney (1)	541.2	633.5
Pituitary	4.0	8.2
Testis (1)	428.0	194.1

the figures for control animals. Determinations were also made for muscle glycogen, but since the values obtained for both groups of animals were in the range for normal rats, the data are not presented.

At autopsy certain organs of the diabetic rats were removed and weighed. The data from these observations are shown in Table 2. Although the animals receiving diethylstilbestrol weighed less at autopsy, the kidneys and pituitaries of treated diabetic rats were larger than those for the controls. The adrenals were not affected markedly by the diethylstilbestrol but the testes showed the usual atrophic condition found after estrogenic treatment.

DISCUSSION

In earlier experiments it was found that in diabetic rats fed ad libitum the administration of diethylstilbestrol caused a decrease in glycosuria, and it was thought that this drug was responsible for the diminution of urinary sugar (Janes and Friedgood, unpublished data). Although there was a decrease in glycosuria in animals studied under the present paired feeding experiments, the data fails to show why this occurred in the treated animals, since their non-fasting blood sugar values remained at the pretreatment level. This might be explained on the basis that in animals treated with diethylstilbestrol there is an increase in the renal threshold for sugar. However, these elevated non-fasting blood sugar values for the treated rats may be

partially due to the fact that in feeding ad libitum some of them actually consumed food just before they were bled. Thus, these higher blood sugar values are not representative of the actual condition of hyperglycemia, and the decreased glycosuria is probably associated with the reduced food intake. That there is some slight permanent elevation in the blood sugar values of the treated animals as compared to the controls was evidenced by the data obtained in the final tests made after a 24 hour fasting period.

The reason the animals treated with diethylstilbestrol lost more weight than the controls when both received the same amount of food is not clearly understood. The treated animals did have slightly higher values for blood sugar than the controls. This suggests that rats treated with diethylstilbestrol were not oxidizing glucose as efficiently, or that they were forming glucose from some endogenous carbohydrate source more rapidly than the controls, probably by gluconeogenesis from protein. Diethylstilbestrol may cause an increase in gluconeogenesis from protein in normal rats by its action through the adrenal cortex (Janes and Nelson, 1942, a). Following the 24 hour fast the N.P.N. values for both the diabetic controls and treated animals in the present experiment were considerably elevated. This elevation was greater in the treated rats, indicating that they were probably utilizing protein more rapidly than the controls. Thus a greater loss of body weight would be expected in the treated animals. However, the non-fasting blood N.P.N. values are not indicative of a difference in the rate of gluconeogenesis. The N.P.N. values presented for the control diabetic rats are higher than those of normal rats (Anderson, Honeywell, Santy, and Pederson, 1930) and more closely approximate values for uncontrolled human diabetics (McDonald, 1939; Collen, 1942).

The data presented do not show that diethylstilbestrol is a diabetogenic agent as was concluded by Ingle and Nezamis (1943) while working with normal and partially pancreatectomized rats. In the present experiment, the animals which received diethylstilbestrol were allowed to feed ad libitum. While receiving this drug they actually ate less food than during the pretreatment period. Ingle and Nezamis, however, force-fed a constant amount of food, rich in carbohydrates, throughout their experiment. In earlier work it was pointed out that diethylstilbestrol caused a slight increase in blood sugar values and that this increase was particularly pronounced for the first few hours after this drug was injected (Janes and Nelson, 1942, b). It is possible that force-feeding would accentuate this hyperglycemic tendency, causing the blood sugar to rise above the renal threshold to produce the mild glycosuria which was described by Ingle and Nezamis. If the animals used in this experiment had been force-fed with the same amount of food that they consumed during the pretreatment period, it is likely that their blood sugar

levels and urinary sugar excretion would have been increased over the values which are reported in this study.

Furthermore, no marked improvement in alloxan diabetes has been seen following the administration of diethylstilbestrol. Although glycosuria was somewhat reduced, the blood sugar, in general, was maintained at the pretreatment level. This is not in agreement with the work of Barnes, Regan and Nelson (1933) and Nelson and Overholser (1936) who administered naturally occurring estrogens to diabetic dogs and monkeys. They found an amelioration of diabetic symptoms following such therapy. Since the dogs were placed on a constant diet, the improvement of diabetic symptoms could not be attributed to a reduced food intake. Although a synthetic estrogenic substance was used in the present experiments and the diabetes was produced by a different method, it appears that the diabetic rat reacts to estrogen treatment in a manner somewhat different than does the dog and monkey. One possible improvement in diabetes caused by diethylstilbestrol is an increased storage of liver glycogen. Except for one pair of rats used in this study, values for liver glycogen were found to be higher in the treated rats than in the controls. The carbohydrates utilized by the treated rats in elevating the liver glycogen above that of the controls may be derived, in part, from an increase in gluconeogenesis from protein. It has been suggested (Janes and Nelson, 1942, a) that diethylstilbestrol stimulates the adrenal cortex in normal rats causing the production of a hormone which promotes gluconeogenesis from protein. Liver glycogen is usually reduced in alloxan diabetes (Lackey, Bunde, Gill and Harris, 1944) and since the treated rats of the present series had high hepatic glycogen levels, it is possible that there may be an improvement in the well-being of these animals, which was not shown in the blood and urine studies.

SUMMARY

Twenty-four alloxan-diabetic rats whose diabetes was stable were paired according to similarity of weights and blood sugar values. Diethylstilbestrol was administered either by injections or the implantation of pellets to one rat of each of the twelve pairs for a period of 21 days. The rats given diethylstilbestrol exhibited slight anorexia so the control mates were limited to the same amount of food eaten by the treated animals. With reduced food intake all of the rats lost weight but this was more pronounced in animals receiving diethylstilbestrol. During non-fasting periods the blood sugar remained at the pretreatment level in animals receiving diethylstilbestrol but decreased somewhat in the controls. The urine volume and sugar decreased in both control and treated rats. Values for blood N.P.N. were similar except at the end of the 24 hour fast when values were increased for both groups, but were the highest in the treated rats. Liver glycogen was markedly increased following treatment with diethylstilbestrol.

From the data presented it is not apparent that diethylstilbestrol is a diabetogenic agent or that it ameliorates diabetic symptoms in the rat. The fall in glycosuria may be associated with reduced food intake.

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METABOLISM OF THE STEROID HORMONES: THE ISOLATION OF AN ANDROGEN FROM HUMAN URINE CONTAINING AN 11-OXYGEN SUBSTITUTION IN THE STEROID RING[1]

A. M. MILLER AND RALPH I. DORFMAN

From the Departments of Medicine and Biochemistry, Western Reserve University School of Medicine and Lakeside Hospital

CLEVELAND, OHIO

AND

ELMER L. SEVRINGHAUS

Department of Medicine, University of Wisconsin Medical School

MADISON, WISCONSIN

IN A RECENT REPORT Mason (1945) announced the isolation of a steroid from human urine which appeared to be androstanediol-3(α), 11(β)-17-one.[2] The compound was isolated from the urines of three women with adrenal tumors, three female pseudohermaphrodites with adrenal hyperplasia, and a young boy with adrenal hyperplasia. We have isolated the same compound from the urine of a young woman suffering from virilism. Further, we have been able to show that this steroid is an androgen and is related to the androgenically active androstenol-3(α)-17-one previously isolated from the urine of the same patient (Dorfman, Schiller and Sevringhaus (1945)). This latter paper also contains the clinical findings on this patient.

EXPERIMENTAL

A total of 76.1 liters of urine was collected with toluene as the preservative. The unhydrolyzed preserved urine was extracted three times with fresh $\frac{1}{4}$ volume portions of benzene. The extraction was carried out between 3 to 9 days after collections were made, and the pH of the urine at the time of extraction varied from about 5 to 6.

The combined benzene extractions representing 76.1 liters were evaporated to dryness and the residue dissolved in 750 cc. of ether and extracted four times with 75 cc. portions of 10% sodium hydroxide. The ether layer was washed with 75 cc. portions of distilled water until the water layer was neutral to litmus. The residue on

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[1] This investigation was supported by grants from the Committee on Research in Endocrinology, National Research Council and Sharpe and Dohme.

[2] In conformity with Dr. Mason we are designating the 11-hydroxy substitution as 11(β).

evaporation of the ether weighed 14.2 grams. This neutral fraction was treated with 18 grams of the Girard-Sandulesco reagent T at room temperature according to the method of Reichstein (1936). In this manner four fractions were separated; fraction A which was extracted with ether from an alkaline solution, fraction B which was extracted with ether from a solution at pH 7, fraction C which was extracted with ether at pH 4.5, and finally fraction D which was extracted with ether from a solution which had been strongly acidified with concentrated hydrochloric acid (1 volume of concentrated hydrochloric acid to 10 volumes of solution at pH 4.5).

Fraction D which amounted to 4.4 grams of material was separated with digitonin (1 per cent digitonin solution in 90 per cent ethanol). The digitonin nonprecipitable fraction which weighed 3.3 grams was acetylated with acetic anhydride and pyridine in a water bath at approximately 90° C. for one hour. The acetylated product, weighing 3.8 grams, was dissolved in 100 cc. of a mixture of benzene and petroleum ether (4:1) and adsorbed on a column of 114 grams of Brockmann's aluminum oxide. The column was eluted with a mixture of benzene and petroleum ether (4:1), benzene, and benzene containing increasing amounts of ether. From fractions representing a total of 1700 cc. of benzene and ether (3:1) crystalline material was obtained. On repeated crystallization from absolute methanol a constant melting point of 238–9° C.[3] was obtained. When this sample was mixed with a sample of the 11-oxygen substituted steroid monoacetate[4] isolated by Mason (1945) M.P. 238–239.5° C. the melting point was 238–239.5° C. More than 800 milligrams of this acetate of melting point above 235° C. has thus far been isolated.

A sample of the monoacetate of androstane-3(α), 11(β)-diol-17-one was heated for one hour at 100° C. at one micron pressure and analyzed.

	C	H
Calculated for $C_{21}H_{32}O_4$	72.38	9.26
Found	72.78	9.56

On saponification of the acetate with sodium hydroxide in alcoholic solution a product melting at 194.5–196.5° C. was obtained. When mixed with a sample of Mason's free compound, M.P. 194.5–196.5° C., the melting point was 194.5–196.5° C.

Twenty-seven milligrams of the androstanediol-3(α), 11(β)-17-one acetate was refluxed ½ hour in one cc. of a mixture of glacial acetic acid and concentrated hydrochloric acid (4:1). The material was taken up in ether, extracted with alkali and finally with water. The residue was reacetylated with acetic anhydride and pyridine. The

[3] Melting points were determined with the Fischer-Johns apparatus and are uncorrected.

[4] We are indebted to Dr. H. L. Mason for samples of both the free compound and monoacetate.

reacetylated material was dissolved in 5 cc. of a mixture of benzene and petroleum ether (1:4) and chromatographed on a column containing 900 mg. of Brockmann's aluminum oxide. The column was eluted with mixtures of benzene and petroleum ether and benzene. Five fractions were obtained which melted at 188.5–189.5° C. after crystallization from absolute methanol. An androstenol-3(α)-17-one was previously isolated from the urine of the same patient. The acetate of this compound melted at 179–82° C. which agreed well with a similar substance isolated by Wolfe, Fieser, and Friedgood (1941). A melting point of the product of the acid dehydration, melting point 188.5–189.5° C., when mixed with the previously isolated androstenol-3(α)-17-one acetate, melting point 179–182° C., was 182–187° C. It was further found that after 5 recrystallizations from absolute methanol the androstenol-3(α)-17-one acetate previously isolated melted at 188.5–189° C. No depression in melting point was found when the recrystallized androstenol-3(α)-17-one acetate was mixed with the acid dehydrated product.

Treatment of the acetylated dehydrated product with alkali in alcoholic solution yielded a substance which melted at 186–187.5° C. When the saponification product was mixed with the starting material, melting point 188.5–.89.5° C., a sharp depression in melting point was found, 153–154° C. There was no depression of the melting point when mixed with a sample of the androstenol-3(α)-17-one, melting point 186–7° C., which had been recrystallized 3 times from aqueous methanol. Previously the melting point of this compound had been reported as 181–181.5° (Dorfman, Schiller and Sevringhaus, 1945).

A second batch of 50 mg. of the acetate of androstane-3(α), 11(β)-diol-17-one was subjected to acid treatment as previously described. A yield of 12.6 mg. of crystalline material melting point 188.9–189.5° C. was again obtained. The mother liquors contained an additional 22.6 mg. of material which after 2 crystallizations from methanol melted at 188–9° C.

A sample of the dehydrated product was dried for 2 hours at 100° C. and 1 micron pressure and analyzed.

	C.	H.
Calculated for $C_{21}H_{30}O_3$	76.31	9.15
Found	76.41	9.37

A bioassay for androgenic activity of the monoacetate on 36 chicks run in parallel with androsterone treated chicks by the method of Frank et al. (1942) indicated activity of the order of one fourth that of androsterone.

DISCUSSION AND CONCLUSIONS

That the compound reported in this communication is the same as that previously isolated by Mason seems quite certain. Mason pre-

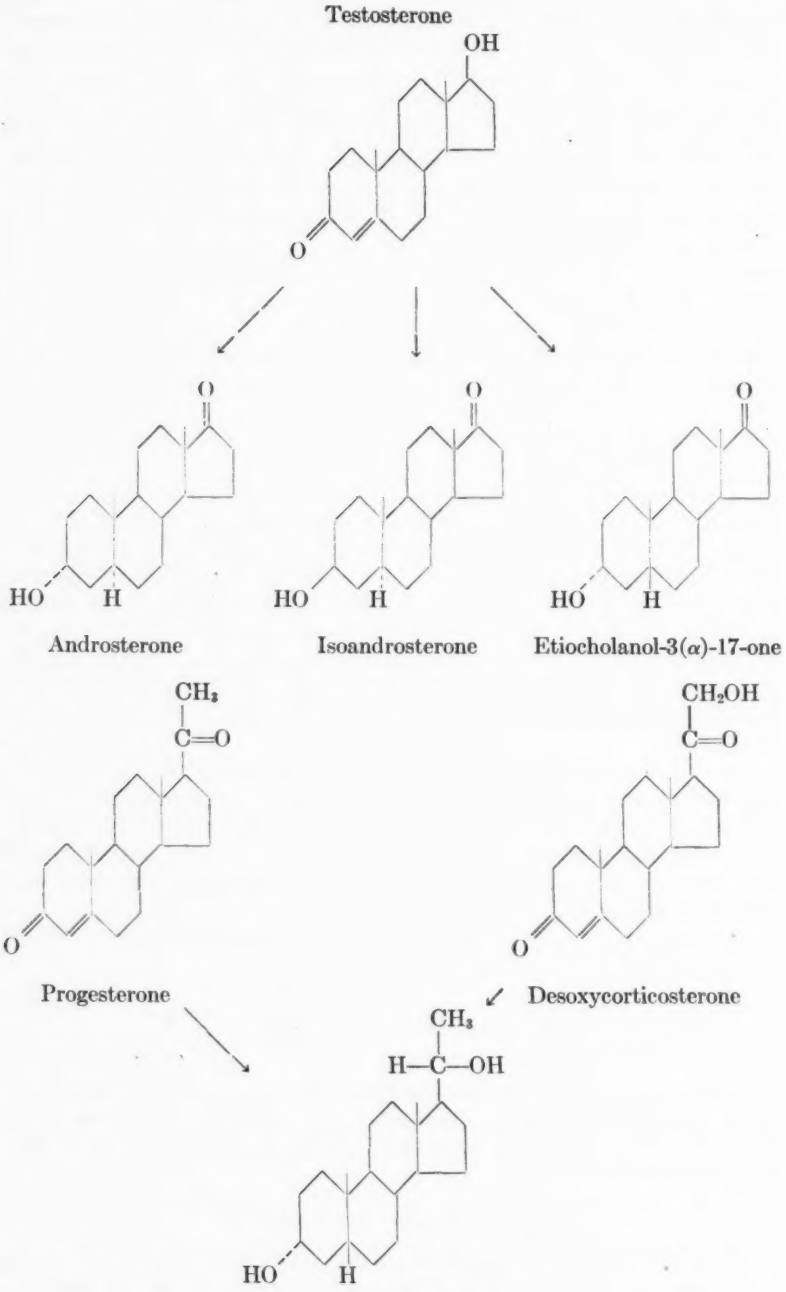

FIG. 1. The metabolism of testosterone, progesterone and desoxycorticosterone.

ferred the placing of a hydroxy group at C-11 to a ketone group even though he pointed out that rigid proof had not as yet been found. The new evidence presented in this communication indicates that the 11 substitution is a hydroxy group since treatment of the monoacetate with a mixture of glacial acetic and hydrochloric acid causes a loss of 1 mole of water. Further, this dehydrated product appears to be identical with the androstenol-3(α)-17-one previously isolated (Wolfe, Fieser, and Friedgood, 1941, and Dorfman, Schiller, and Sevringhaus, 1945).

These experiments also help to narrow the possibilities as to the position of the double bond in androstenol-3(α)-17-one. Wolfe, Fieser, and Friedgood (1941) suggested that the double bond was between carbon atoms *6:7*, *7:8*, *9:11*, or *11:12*. It now appears that the double bond is at either 9:11 or 11:12. That the unsaturated material is a mixture of both 9:11 and 11:12 isomers is not ruled out at this time.

The finding of the androstane-3(α), 11(β)-diol-17-one in the unhydrolyzed urine and its dehydration by treatment with acid strongly suggest that the androstenol-3(α)-17-one previously isolated from the hydrochloric acid hydrolyzed urine of the same patient is a degradation product of the 11-hydroxy compound.

In view of the mild procedures employed it appears quite likely that the androstanediol-3(α), 11(β)-17-one isolated is a true metabolite of the adrenal cortical steroids. This would indicate a degradation of a C21 steroid to a C19 steroid. A direct transformation of this type has not as yet been realized. The indicated change in ring A is one which has been previously demonstrated for testosterone (Callow, 1939; Dorfman, Cook, and Hamilton, 1939; Dorfman, 1941), progesterone (Venning and Browne, 1937), and desoxycorticosterone (Westphal, 1942; Fish, Horwitt, and Dorfman, 1943; Horwitt, Dorfman, Shipley, and Fish, 1944; Hoffman, Kazmin, and Browne, 1943), that is, reduction of the 3-ketone to the 3-hydroxy and the saturation of the nuclear double bond. The spatial configuration at C-5 has been shown to be both allo and normal for the metabolites of testosterone while for the metabolite of progesterone and desoxycorticosterone only the normal C-5 configuration has been found (Fig. 1). Metabolism of testosterone has yielded compounds with configurations at C3 of both 3(α) hydroxy and 3(β) hydroxy while metabolism of progesterone and desoxycorticosterone have yielded only the 3(α) hydroxy configuration. In this case we find the 3(α) hydroxy C5 allo configuration presumably resulting from the metabolism of an α, β-unsaturated ketone (Fig. 2). Thus it is analogous to the production of androsterone from testosterone. Whether other isomers exist which would be analogous to the isomers of androsterone is not known at this time.

It is interesting to note that both the acetates of the androstenol-3(α)-17-one and the C-11 oxygen compound androstanediol-3(α), 11 (β)-17-one described here possess androgenic activity of the same order, that is about one fourth the activity of androsterone.

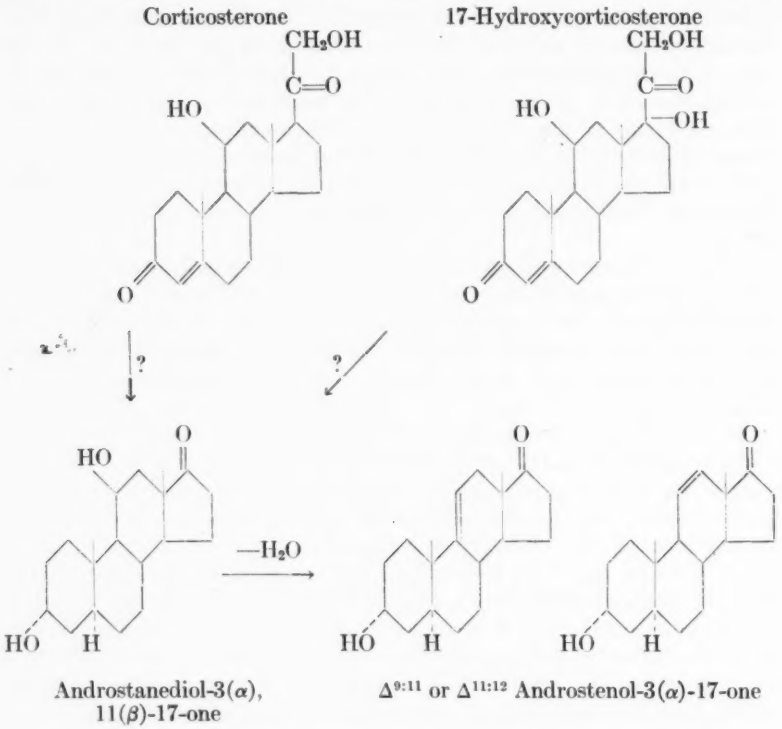

FIG. 2. Possible metabolism of corticosterone and 17-hydroxycorticosterone.

The yield of androstanediol-3(α), 11(β)-17-one isolated was approximately 800 mg. which would be of the order of 10.5 mg. per liter.

SUMMARY

Ten and five tenths (10.5) milligrams per liter of androstanediol-3(α), 11(β)-17-one have been isolated from the unhydrolyzed urine of a girl suffering from virilism. This compound appears to be identical with a C-11 oxygen substituted steroid previously isolated from human urine by Mason.

The androstanediol-3(α), 11(β)-17-one has been converted by dehydration into an androstenol-3(α)-17-one apparently identical with a substance previously isolated from hydrochloric acid hydrolyzed urine from the same patient. This conversion definitely establishes the presence of an 11-hydroxy substitution in the 11-oxygen compound and also narrows the possibilities of the unsaturation in the androstenol-3(α)-17-one to the 9:11 or 11:12 position.

It has been demonstrated that androstanediol-3(α), 11(β)-17-one

is approximately one fourth as active as androsterone as measured by a chick comb method.

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THE EFFECT OF DIET ON THE GROWTH AND SURVIVAL OF ADRENALECTOMIZED RATS TREATED WITH DESOXYCORTICOSTERONE ACETATE PELLETS[1,2]

ALBERT SEGALOFF[3]

From the Department of Medicine, Vanderbilt University, School of Medicine
NASHVILLE 4, TENNESSEE

INGLE, GINTHER AND NEZAMIS (1943) have demonstrated that the level of carbohydrate in the diet has no significant effect on growth or duration of survival of adrenalectomized rats either untreated or given 1 per cent sodium chloride in the drinking water. However, Swann (1937) found that the addition of bread to the diet of adrenalectomized rats greatly prolonged their survival. Long and his co-workers (1940, 1942) have presented much evidence that the C 11 oxygenated steroids promote the production of liver glycogen in the fasted, adrenalectomized rat but that large amounts of desoxycorticosterone acetate do not lead to glycogen deposition in their animals. The various aspects of this problem have recently been reviewed by Swingle and Remington (1944) and by Ingle (1944).

The present studies were undertaken to ascertain the effect of diets free from carbohydrate and fat and the effect of the quality of protein on the growth and survival of adrenalectomized rats.

MATERIAL AND METHODS

Immature male rats, purchased from Maguran Farms, Birmingham, Michigan, ranging from 40 to 66 gm. in weight and averaging 50 gm. were used. They were twenty-eight to thirty days old at operation. Four diets were employed; their composition is shown in Table 1.

The fatty acids were prepared by saponification of a single lot of hydrogenated vegetable shortening (Crisco). The soaps were extracted with ether and acid precipitated. S.M.A. Co. vitamin free casein was employed. The crude casein (H.I.P.) was purchased from the Casein Company of America.

The pellets of desoxycorticosterone acetate were 3 mm. in diameter and weighed from 18 to 27 mg. They were prepared in a simple die and compressed in a hand arbor press.

Adrenalectomy was performed under ether anesthesia; both adrenal

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[2] Presented before the 18th annual meeting of the Central Society for Clinical Research, Chicago, Nov. 2, 1945.
[3] Present address: The Alton Ochsner Medical Foundation, New Orleans 15, Louisiana.

glands were removed at a single operation through one dorsal skin incision. The pellets were implanted through the same incision in the subcutaneous tissue between the scapulas.

The animals were placed in individual, wire bottom cages and fed the experimental diets immediately after operation. The diets and tap water were constantly available. The few animals which survived and grew after removal of the pellets and return to the stock diet of Purina dog chow were

TABLE 1. COMPOSITIONS OF DIETS. ALL EXPRESSED ON BASIS OF 100 GMS. OF DIET

	Diet 1	Diet 2	Diet 3	Diet 4
Casein (S.M.A. Co. Vitamin free)[1]			80 gm.	95 gm.
Casein[2]	80 gm.	95 gm.		
Salt mixture (U.S.P. XII)	5 gm.	5 gm.	5 gm.	5 gm.
Fatty acid mixture	15 gm.		15 gm.	
Thiamin chloride	0.8 mg.	0.8 mg.	0.8 mg.	0.8 mg.
Riboflavin	1.6 mg.	1.6 mg.	1.6 mg.	1.6 mg.
Calcium pantothenate	1.0 mg.	1.0 mg.	1.0 mg.	1.0 mg.
Nicotinic acid	2.0 mg.	2.0 mg.	2.0 mg.	2.0 mg.
Pyridoxin	1.2 mg.	1.2 mg.	1.2 mg.	1.2 mg.
Choline chloride	400.0 mg.	400.0 mg.	400.0 mg.	400.0 mg.
α-Tocopherol	10.0 mg.	10.0 mg.	10.0 mg.	10.0 mg.
Vitamin A[3]	2400 U.S.P. units	2400 U.S.P. units	2400 U.S.P. units	240 U.S.P. units
Vitamin D[33]	240 U.S.P. units	240 U.S.P. units	240 U.S.P. units	240 U.S.P. units

[1] Now marketed by General Biochemical Inc. as Casein (G.B.I.).
[2] H.I.P. casein. The Casein Corp. of America.
[3] Desynon-Winthrop Chemical Co. Inc.

omitted from the summary since they all had macroscopic accessory tissue at autopsy. Nine rats were given diet 1; 15, diet 2; 37, diet 3; and 17, diet 4.

RESULTS

The gain in weight is shown in figure 1, and the duration of survival of the animals on each diet is presented in figure 2. It can be seen that neither group fed the diets free from both carbohydrate and fat (diets 2 & 4) survived an appreciable period. A few of the rats fed the diet containing the vitamin free casein plus the fatty acid mixture (diet 3) survived as long as 30 days but, as can be seen from figure 1, their growth was very slow during this period of survival. The animals fed the crude casein plus the fatty acid mixture (diet 1) all survived and grew well until the removal of the pellets on the 58th day. A daily average of 0.18 mg. was absorbed from each pellet.

DISCUSSION

The results obtained raise several questions which we are unable to answer with our present state of knowledge. The vitamin supplements were those required for growth in the rat (Richardson et al. 1941); thus inositol, biotin and para-aminobenzoic acid, which are

necessary for adequate lactation and reproduction, were omitted and may be required for the growth of the adrenalectomized rat in our experiments. The crude casein might contain some unknown factor or there might be sufficient carbohydrate in the casein to account for the phenomenon.

The fatty acids may be necessary in that the animal may be converting them directly into glycogen, as has recently been demon-

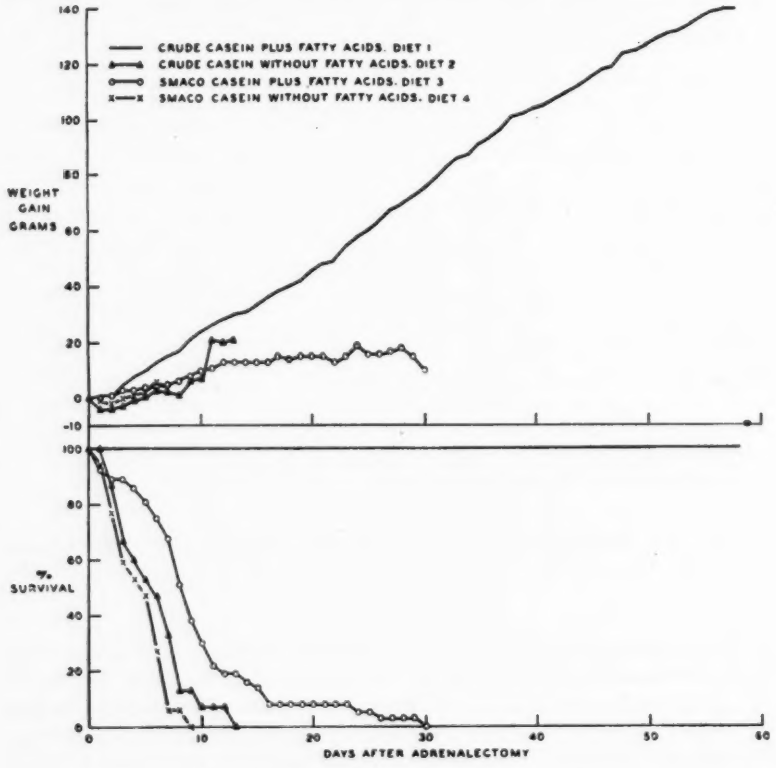
CRUDE CASEIN PLUS FATTY ACIDS. DIET 1
CRUDE CASEIN WITHOUT FATTY ACIDS. DIET 2
SMACO CASEIN PLUS FATTY ACIDS. DIET 3
SMACO CASEIN WITHOUT FATTY ACIDS. DIET 4
WEIGHT GAIN GRAMS
% SURVIVAL
DAYS AFTER ADRENALECTOMY

FIG. 1. Growth of adrenalectomized rats on various diets.
FIG. 2. Survival of adrenalectomized rats on various diets.

strated in the intact animal fed sodium butyrate by Dziewiatkowski (1944), or the "essential" fatty acids may be necessary as a catalyst for gluconeogenesis.

Thus, it would appear that although desoxycorticosterone acetate is not capable of producing excess liver glycogen in the partially adrenalectomized rat (Long, et al. 1940, 1942; Olson et al. 1944), it is capable of supporting gluconeogenesis to an extent sufficient to produce good growth and survival on a diet without carbohydrate but

containing fatty acids, salts, vitamins and crude casein. Or alternately the high protein diet may supply sufficient extra protein to overcome the defect since it has been demonstrated by Lewis et al. (1940) that the adrenalectomized rat can convert some injected alanine to glucose.

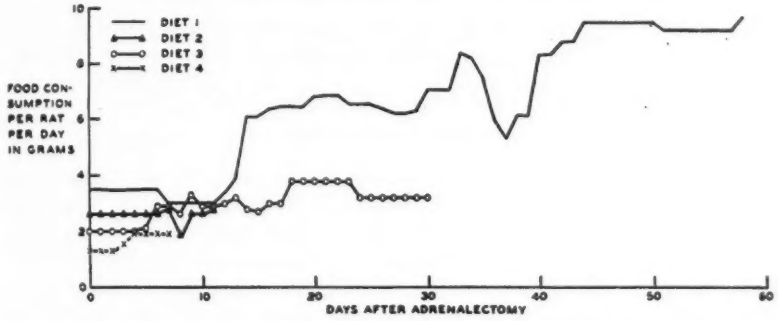

FIG. 3. Food consumption of adrenalectomized rats on various diets.

CONCLUSION

Adrenalectomized rats with subcutaneous pellets of desoxycorticosterone acetate grow and survive on a diet free from carbohydrate but containing crude casein, fatty acids, minerals and synthetic vitamins. Growth and survival are greatly diminished when a purified casein is substituted or the fatty acids are omitted.

ACKNOWLEDGEMENT

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THE INFLUENCE OF ADRENALECTOMY ON THE UTILIZATION OF ACETONE BODIES[1]

EATON M. MacKAY, ARNE N. WICK
AND CYRUS BARNUM

From The Scripps Metabolic Clinic
LA JOLLA, CALIFORNIA

BECAUSE adrenalectomy reduced the ketonuria we concluded (MacKay and Barnes 1936, 1937) along with others (Evans, 1936 and Fry 1937) that this procedure had an antiketogenic action, reducing acetone body formation in the organism. Subsequent studies (Mirsky 1938, Shipley and Long 1938, and MacKay and Wick 1939) have shown that the changes in the ketonuria were due to changes in the renal threshold for the acetone bodies. The same observations invalidate the suggestion we once made (MacKay and Barnes 1938) that adrenalectomy increases the ketolytic activity of the organism. This has already been pointed out (MacKay and Wick 1939) where we concluded that "The influence of adrenalectomy upon ketolytic activity will have to be studied by the use of arterio-venous differences. . . ." The results of such a study are presented here.

METHODS

Adult albino rats of quite large size were used so that adequate blood specimens could be obtained. The adrenalectomized groups had their adrenals removed under ether anesthesia through a single skin incision in the back and a small muscle slit on either side over the kidney. They had completely recovered within 5 minutes after the administration of the anesthetic was discontinued. The control groups were subjected to a similar but sham operation in which the adrenals were not removed.

Except in one experiment (No. 10) the ketosis was of endogenous origin and developed as a result of fasting. Throughout the period after adrenalectomy 0.8 per cent NaCl was allowed *ad libitum* as the drinking fluid. At the completion of the experimental period the rats were anesthetized by an intraperitoneal injection of sodium pentobarbital and slightly more than 1 cc. of blood drawn from the inferior vena cava well below the entrance of the renal veins. A similar sample was then taken from the abdominal aorta as quickly as possible. Equal volumes of arterial and venous blood from each rat of the groups of 3 rats (6 in Exp. 10) were then pooled to provide large enough specimens for satisfactory analyses. Blood acetone body determinations were made by the method of Barnes and Wick (1939). Blood sugar determinations were carried out on some specimens according to Shaffer and Somogyi (1933).

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[1] The experiments comprising this study were carried out in 1940.

RESULTS

The data summarized in Table I show very clearly that adrenalectomy produces a definite increase in the rate of acetone body removal from the blood in fasting rats. The direction of the change in the arterio-venous difference is uniform throughout the fasting groups although the amount of the change is irregular. This would be expected if the mechanism we believe responsible for the increased ketolysis after adrenalectomy is correct. In some of the experiments (Nos. 2, 5, 6, and 7) the higher arterio-venous acetone body differences after adrenalectomy might be explained in part at least by the higher acetone body level of the blood in the animals with the glands removed. However, the average increase in the arterio-venous difference is practically as high in those experiments (Nos. 1, 3, 4, 8, and 9) in which the acetone body level was essentially the same in both groups.

If adrenalectomy had reduced the arterio-venous acetone body difference the oxygen utilization of the tissues served by the vessels from which our specimens were obtained would be of interest because of the well-known impairment in the circulation which is apt to occur after adrenalectomy. In the present case we may neglect it.

The arterio-venous blood sugar differences which are recorded may be satisfactory for purposes of comparison but unlike the acetone body values do not have an absolute value in the rat. The reason for this is that the anoxia which frequently develops as a result of taking the venous specimen leads to an outpouring of such glycogen as there may be in the liver giving arterial blood sugar values which are too high.

DISCUSSION

The most likely explanation of the increased "utilization" of acetone bodies following adrenalectomy is the one which we advanced in an earlier paper (MacKay and Barnes 1938) leading to this same conclusion which we have since shown was not justified on the basis of the experimental method used. Quoting this, "The increased ketolytic activity of the organism which we have demonstrated here is none the less important. The fasting adrenalectomized animal has a low blood sugar and a deficient formation of glucose from lactic acid and protein sources. It is possible that with this dearth of readily oxidizable carbohydrate upon which the organism is ordinarily so dependent that it turns to the ketone bodies when they are available for quick energy yielding substances. It is interesting in this connection that ketone bodies are always produced under those circumstances when there is an absolute lack of glucose or a relative deficiency of oxidizable glucose available for the body economy. It is hoped that the new blood ketone method which has proved suitable for the study of arterio-venous differences may make it possible to determine the purposefulness of a ketosis in supplying oxidizable fuel of the order of glucose when the latter is unavailable."

TABLE I

Exp. no.	No. rats in each group	Sex	Hours fasting	CONTROLS							ADRENALECTOMIZED								Deviation from control A-V acetone body difference mg. %
				Avg. body weight gm.	Blood Acetone Bodies			Blood Sugar			Av. body weight gm.	Hours after adrenalectomy	Blood Acetone Bodies			Blood Sugar			
					Arterial mg. %	Venous mg. %	A-V diff. mg. %	Arterial mg. %	Venous mg. %	A-V Diff. mg. %			Arterial mg. %	Venous mg. %	A-V Diff. mg. %	Arterial mg. %	Venous mg. %	A-V Diff. mg. %	
1	3	♀	48	228	9.00	8.76	0.24	98	80	18	222	48	10.40	9.33	1.07	58	58	0	+0.83
2	3	♀	48	191	9.56	7.72	1.84				192	48	16.20	14.20	1.90	77	67	10	+0.06
3	3	♂	48	256	12.43	11.08	1.35	66	54	12	260	24	15.92	13.76	2.16	66	59	7	+0.81
4	3	♂	24	277	3.10	2.77	0.33				270	24	3.29	2.38	0.91				+0.58
5	3	♂	48	312	7.70	6.90	0.60	88	70	18	309	48	10.80	8.50	2.30	58	48	10	+1.70
6	3	♂	96	314	10.00	9.20	0.80	78	67	11	310	48	20.30	18.50	1.80	56	54	2	+1.00
7	3	♂	24	320	10.70	8.25	2.45				318	120	17.10	13.80	3.30				+0.85
8	3	♂	24	319	10.10	8.80	1.30				319	120	10.90	9.04	1.86				+0.56
9	3	♂	24	316	11.10	10.60	0.50				320	120	9.20	8.28	0.92				+0.42
10[1]	6	♀	0	224	10.61	9.38	1.23	105			222	24	10.99	9.85	1.14				−0.07

[1] Increase in the blood acetone bodies produced by feeding the sodium salt of B-hydroxybutyric acid.

Additional evidence that adrenalectomy increases the "utilization" of ketone bodies by the tissues during fasting because of the paucity of glucose is seen in the smallest increase in the A-V acetone body difference in the experiment (No. 2) in which the highest blood sugar was recorded. Furthermore the only negative deviation in the A-V difference after adrenalectomy was observed in fed animals (Exp. 10). These rats had been given glucose by stomach tube from time to time in 50 per cent solution and were given 0.5 cc. per sq. dm. body surface of 1 molar sodium B-hydroxybutyrate by stomach tube 3 hours and 1 hour prior to making the observations which are recorded.

The conclusions which are presented here appear to be directly opposed to those reached by Nelson, Grayman and Mirsky (1940) on the same question. The method which they used for studying acetone body utilizations necessitated the use of fed rats, without ketosis and with adequate amounts of sugar available. In the single experiment in our study carried out with fed rats (No. 10) we found no increased utilization such as occurs in fasting. But Nelson et al. report a decrease in acetone body utilization after adrenalectomy. We suspect that the decrease found in their experiments was due to the heroic treatment (100 mg. glucose per 100 gm. body weight intraperitoneally 80 minutes and 0.85 Mm. sodium B-hydroxybutyrate per 100 gm. body weight intravenously 40 minutes before the rats were killed) which the rats received and which would be bound to be much more severe on the adrenalectomized rats. In our experience such treatment would reduce all of the body functions in the adrenalectomized organism. We might point out that our adrenalectomized rats were all in excellent condition.

There is still another possible explanation for the different conclusion reached by Nelson, Grayman and Mirsky and ourselves. The former group was measuring acetone body utilization by the entire animal but we are measuring utilization as the quantity of acetone bodies removed from the blood stream by a part of the body tissues. If, as is apt to happen, the metabolic rate and hence blood flow and oxygen utilization of the tissues were reduced in our adrenalectomized animals, it might well be that our choice of method for measuring utilization may be responsible for the diverse conclusion reached by Nelson et al. and ourselves. Since we are actually concerned with acetone body "utilization" in relation to the total energy requirement of the control and adrenalectomized organism respectively, it is probable that our method provides a better measure of utilization by the use of the entire animal unless the total metabolism is simultaneously measured. Nelson et al. recognized this difficulty with their method and state, ". . . it is probable that the removal of the adrenals results in a decreased acetone body utilization in virtue of the general decrease of metabolic activity that ensues." We are interested in a different type of utilization in the present study.

During fasting adrenalectomized rats "utilize" acetone bodies more readily than normal animals and as the level of acetone bodies is not appreciably lower in the blood than in normals, it stands to reason that there is an increased ketogenesis after adrenalectomy. The acetone body level in the blood would then be apt to be higher than normal in adrenalectomy and this does occur (Exps. 2, 5, 6, and 7 in the present study). In the absence of an adequate sodium balance or the result of measures which the adrenalectomized organism is unable to resist and the onset of varying degrees of so-called "adrenal intoxication" there is a general depression of metabolism and the acetone body level in the blood is lowered probably a result of diminished ketogenesis as suggested by Nelson et al. (1940).

SUMMARY

Adrenalectomy produces an increase in the rate of acetone body utilization in the fasting rat with a ketosis when this is measured by arterio-venous differences. It is suggested that the deficiency in available glucose in this condition is responsible for the increased ketolysis.

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THE ROLE OF THE CORPORA ALLATA IN THE DEVELOPMENT OF LEUCOPHAEA MADERAE (ORTHOPTERA)[1]

BERTA SCHARRER

From the Department of Anatomy, Western Reserve University
CLEVELAND, OHIO

IT IS WELL KNOWN that the corpora allata play an important role in insect development. It is also established beyond doubt that this influence is hormonal in nature. There is, however, no full agreement among investigators as to the mode of action of the corpus allatum hormone and its interaction with other factors in the control of the various developmental steps. The present paper is intended as a contribution to the study of these problems.

MATERIAL AND METHODS

The insect used, Leucophaea maderae, is an ovoviviparous roach belonging to the subfamily Panchlorinae. The animals were bred in the laboratory at room temperature. Stock colonies were kept in large earthenware crocks; individuals or small groups of experimental animals and controls were isolated in glass jars of pint or two quart size. The animals lived well on fresh carrots alone, but from time to time this diet was supplemented by hard-boiled eggs.

Under these laboratory conditions Leucophaea was found to have on the average eight nymphal instars preceding the emergence of the adult (fig. 3, A). As has also been reported with respect to other roaches (Gould, 1941; Griffith and Tauber, 1942), the time elapsing between consecutive molts may vary considerably. Intermolt periods may take from three to five weeks with the exception of the last which is usually longer (five to eight weeks). Much longer intermolt periods may occur however.

So far no method has been devised to identify with absolute certainty the various instars on the basis of their size and appearance. Therefore, in order to obtain "dated" animals for the operations and controls, nymphs were reared separately from the day of hatching and were observed daily to record the number of molts. Allatectomy was performed in fifth, sixth, seventh, and eighth (last) instar nymphs of both sexes. As the procedure of dating is by necessity rather tedious, it was employed in a limited number of animals only. The data thus obtained were supplemented by operations in nymphs from stock colonies; in these cases the approximate stage was estimated. In all of these animals the date of the molt preceding the operation was recorded.

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For the removal of the corpora allata the nymphs were immobilized with pins and suitable pieces of soft tissue paper on a Petri dish filled with paraffin. No anesthetic was necessary. In order to free the dorsal neck region for the operation a transverse cut had to be made in the anterior portion of the pronotum so that a flap could be folded laterally. This accounts for the slight irregularity in the exoskeleton of this area after molting, visible in figures 1, b and 2, a. For the extirpation of the corpora allata fine forceps and iridectomy scissors were used. In this operation it was impossible to avoid the removal of a small portion of the corpora cardiaca which, in the species studied, are in direct contact with the corpora allata.

In many instances the completeness of the operation was checked by the examination of serial sections through the head region of the allatectomized animals. The material was fixed in Zenker-formol, embedded in paraffin, sectioned 7μ thick, and stained with Foot's modification of Masson's trichrome stain.

RESULTS

If nymphs of Leucophaea maderae are allatectomized successfully in stages earlier than the last within one week after the preceding molt, a disturbance of normal development occurs which is characterized by the suppression of one or more molts. In other words, the operated nymphs undergo their final molt at an earlier stage than normal control animals. Consequently the resulting insects are smaller than normal adults. As they exhibit adult-like characters to a greater or less degree, they are called in this paper "adultoids."

Aside from their reduced size these adultoids are characterized by the relative shortness of their wings. As seen in figure 1, b the wings of the operated animal do not entirely cover the abdomen. Frequently they appear even shorter than in the specimen illustrated. This seems in part due to an incomplete expansion of the wings at emergence. Such wings are ruffled and sometimes frayed at the posterior end; this condition makes it difficult to estimate their true length. They are often oriented more laterally than normal wings and do not overlap. Wigglesworth (1936, plate 5, figs. 13–15; Rhodnius) and Pfeiffer (1945, plate 1, figs. 5, 6; Melanoplus) pictured similar wings in "intermediate" forms which, likewise, were the result of experimentally changed hormone balance. The pronotum of adultoids is adult-like in shape and pattern. The cuticle has a smooth, glossy appearance like that of the normal imago and is distinctly different from the more dull, rough appearance in the nymph. Owing to the shape, size and relative proportions of compound eyes, ocelli, etc., the head resembles that of an adult rather than that of a nymph. Likewise, the posterior abdominal sterna are differentiated according to the adult pattern. Finally, the reproductive organs of male and female adultoids show a degree of development not found in nymphs of comparable stage, but approaching that of adults. They are relatively smaller in size and they may show various degrees of imperfection.

Of the 94 adultoids studied a certain number had difficulties in

emerging properly and died soon afterwards (see also Pfeiffer, 1945, p. 493). In another group the time of survival was obviously shortened by tumors which developed not due to the removal of the corpora allata but due to the incidental cutting of the recurrent nerve at allatectomy (Scharrer, 1945). The remaining adultoids were viable and were observed in the laboratory for many months, some over a year, without any further molt occurring. The life expectancy of these surviving adultoids is, therefore, comparable to that of normal adults.

As may be expected from the known role of the corpora allata in

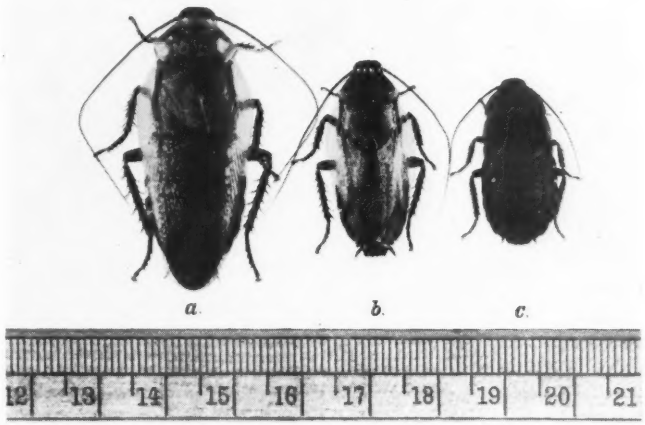

FIG. 1. a. Normal adult male of Leucophaea maderae. b. Adultoid male obtained from allatectomized seventh instar nymph. c. Normal male eighth instar nymph. Scale in centimeters.

the adult female (Scharrer, 1946) egg maturation never took place in an adultoid.

In all dated nymphs that had been allatectomized in the *seventh stage* (fig. 3, C; six specimens) the emergence of adultoids occurred without any further molt. The same result was obtained in 27 additional specimens that, at the time of operation, were estimated seventh instars. Thus, in this series allatectomy at the stage preceding the last, suppressed one molt as compared with normal development.

From *sixth or fifth instar* nymphs (fig. 3, F, G) whose corpora allata were removed successfully (13 dated sixth instars, seven dated fifth instars, 15 estimated sixth or fifth instars) adultoids did not emerge immediately but only after one intervening molt. This molt, following the operation, produced intermediate forms which, although distinctly different from normal nymphs, did not have all the

characteristics of adultoids. Such a "preadultoid" (fig. 2, a) has a smooth and glossy cuticle and a shape that is more imaginal than nymphal in character. Wings are not yet developed, but the wing buds may be quite pronounced. With the next molt these preadultoids invariably became adultoids which fact indicates that in operated sixth instars there had been one, in fifth instars two molts suppressed when the final molt occurred.

FIG. 2. a. Preadultoid obtained from allatectomized sixth instar nymph. Note glossy cuticle, pronounced wing buds. b. Normal seventh instar control nymph. Scale in centimeters.

The adultoids emerging from preadultoids had difficulties in freeing themselves faultlessly from the old cuticle, and therefore did not survive long. In some, but not all cases, the molt or molts following allatectomy were delayed as compared with normal controls. However, since similar delays may be observed in Leucophaea under a variety of experimental conditions, no specific significance can be attributed to this fact.

The smaller the nymphs are, the more difficult is the complete extirpation of both corpora allata. It was not unexpected, therefore, that a number of animals did not reveal any effect of the operation, but continued their development like normal nymphs. Such specimens, in addition to adultoids and preadultoids, were checked histologically and were used as controls. A study of complete serial sections through the head region of both groups of experimental animals furnished this conclusive evidence: When ordinary molts had

continued to occur after the operation, the extirpation was found incomplete. On the other hand, in all cases of total removal of the corpora allata adultoids had resulted.

The corpus allatum, therefore, safeguards the proper number of nymphal molts before the completion of metamorphosis and the emergence of the adult insect. If this gland, i.e. its hormone, is absent during earlier stages of development, the normal balance be-

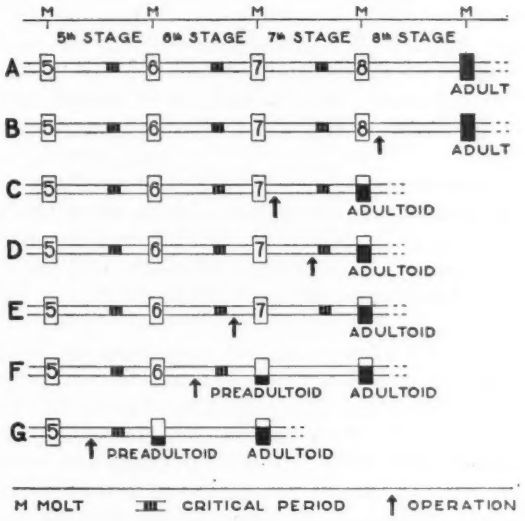

FIG. 3. Diagram summarizing normal and experimentally altered development of Leucophaea maderae. A. Normal development: adult emerges from eighth instar nymph. B. Allatectomy in eighth stage: no visible effect on development. C, D. Allatectomy in seventh stage at various intervals before critical period: next molt is last and results in adultoid. E. Allatectomy in sixth stage after critical period: next molt produces nymph, second molt after operation produces adultoid. F. Allatectomy in sixth stage before critical period: next molt produces preadultoid, second (last) molt adultoid. G. Allatectomy in fifth stage before critical period: next molt produces preadultoid, second molt adultoid. Two molts suppressed.

tween growth and imaginal differentiation is disturbed. Molts are suppressed and the development of adult characters is speeded up, so that a certain degree of malformation may result in some organs, such as wings or reproductive organs.

If absence of corpus allatum results in precocious development, no effect should be expected and none was observed after extirpation of the glands in the last, i.e. *eighth nymphal instar* (fig. 3, B). Eight males and ten females in which the operation had been performed shortly after the preceding molt emerged normally. This result is in agreement with that of Pfeiffer (1939) and of Bounhiol (1939). The females differed from normal ones only in that no egg maturation

occurred (see also p. 37). Four of the males were capable of fertilizing the eggs of virgin females (Scharrer, 1946, p. 53).

It may be concluded that under normal conditions either no or not enough corpus allatum hormone (juvenile hormone) is released during the last nymphal stage. If this hormone is introduced by implantation of corpora allata into last instars supernumerary molts resulting in "giant" forms are obtained. This was reported by Wigglesworth (1934, 1936), Pflugfelder (1939), Pfeiffer (1942, 1945) and Piepho (1942). Preliminary observations in Leucophaea corroborate these findings. Apparently in these experiments the juvenile factor, by suppressing adult differentiation, enabled last instar insects to undergo additional molts. This should not be understood to indicate that the corpus allatum is the source of a molting hormone (see discussion, p. 42).

In the experiments reported so far the corpora allata were removed within a short period, i.e. one to seven days, after the animals had molted. In another series of experiments allatectomy was performed after longer periods, i.e. nine days and more, had passed since the preceding molt. These experiments were based on the following considerations.

The duration of a given nymphal stage may be divided into (1) a period during which the hormone concentration in the circulation is too low to be effective, and (2) a period in which a sufficient hormone level has been reached. Both periods are connected by a "critical period" (Wigglesworth, 1934). In order to study the effect of allatectomy it is necessary to operate at a time when no effective amount of corpus allatum hormone has yet been released into the circulation. In other words, only if the extirpation takes place before the onset of the critical period can it be expected to become fully effective at the following molt (fig. 3, C, D, F, G). On the other hand, allatectomy after the critical period, in all stages but the last, must lead to another nymphal molt. In such cases the effect of allatectomy carried out shortly before the next molt, will manifest itself at the second molt after the operation (fig. 3, E). Thus varying the time of operation within a given stage will furnish information regarding the onset of the critical period.

For experiments of this kind Leucophaea is favorable because of the relatively long duration of nymphal stages. However, the considerable range of individual variation of the intervals between consecutive molts constitutes a disadvantage.

Seventy eight nymphs (instars five, six, and seven) were allatectomized with intervals between preceding molt and operation ("preoperative period") ranging from nine to 49 days. At the molt following the operation 46 nymphs became adultoids, or preadultoids respectively, the rest remained nymphs. In Figure 4 these 78 cases are arranged according to the length of the interval between operation

and following molt ("postoperative period"), ranging from a specimen that molted one day after allatectomy to one that took 56 days. Only the animals above line M (black columns) showed adultoid characters.

In this "adultoid group" the preoperative period ranged from nine

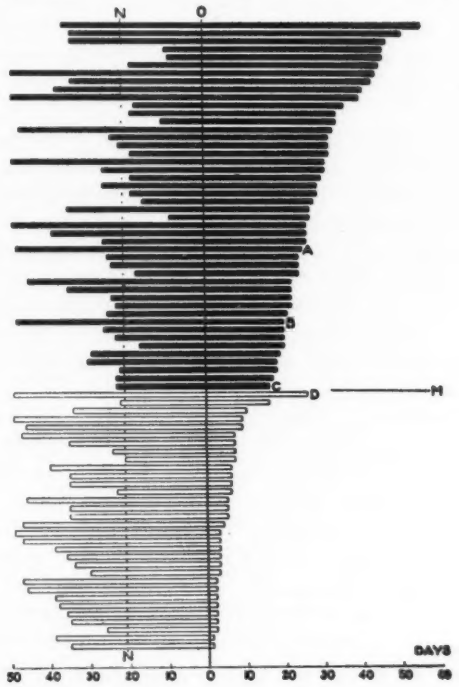

FIG. 4. Diagram summarizing results of allatectomy in nymphs at various intervals. Each column represents one animal. The total length of each column indicates the duration of the intermolt period. This period is divided by the vertical line O (time of allatectomy) into a preoperative period (interval between preceding molt and operation) and a postoperative period (interval between operation and following molt). The arrangement is according to the length of the postoperative periods. The group above line M (black columns) developed adultoid characters, the group below (white columns) remained nymphs at the molt following the operation. A, B, C, D, and N are explained in text, p. 42.

to 49 days, the postoperative period from 16 to 56 days. Irrespective of the actual duration of the entire intermolt period the most instructive cases are those in which the values for the preoperative period are relatively high and those for the postoperative period relatively low. A few such cases are indicated in Figure 4, A, B, C. From these representative cases it is evident that allatectomy is still effective after more than half of a nymphal stage has passed (see especially

cases A and B), and that a minimum of 16 days is still sufficient to change the character of an insect from nymphal to adultoid (case C).

The animals below line M (white columns) retained their nymphal characters at the first molt, and became adultoids at the second molt following the allatectomy. In this group the preoperative period ranged from 21 to 49 days, the postoperative period from one to 26 days. Figure 4 shows that with the exception of case D (which also had the longest intermolt period in the group) the length of the postoperative periods in the nymphs remains well below the values for adultoids.

On the other hand, the relative duration of the preoperative period in the "nymphal group" is greater than in the "adultoid group." All nymphs allatectomized less than 21 days (see line N, fig. 4) after they had molted became adultoids at the next molt. All animals with a postoperative period of less than 16 days remained nymphs at the next molt; all except one, with a postoperative period of over 16 days became adultoids. These time relationships indicate that the period at which, within a given stage, the critical level of the corpus allatum hormone is reached (critical period) lies near the beginning of the second half of the intermolt period.

Animals which were allatectomized shortly before the critical period (fig. 3, D) and consequently had a relatively short postoperative period, showed somewhat less developed adultoid characters than those operated on shortly after they had molted (fig. 3, C). In spite of these minor variations, however, the adultoids could be readily distinguished from nymphs. In other words, the type of response, nymphal or adultoid, changes rather abruptly at the critical period. This result differs in degree from that of Wigglesworth (1934) who obtained in Rhodnius a series of intermediate forms between nymphs and adults by varying the time of operation within a nymphal stage.

DISCUSSION

The specific role of the corpus allatum in insect development as the source of a juvenile (inhibitory) hormone was first postulated by Wigglesworth in his classical work on Rhodnius (1934, 1936, 1940): Acting differently from the "molting hormone" of the brain, the active principle of the corpus allatum restrains adult differentiation and permits the occurrence of further molts. This action is believed to consist in the activation of the nymphal at the expense of the imaginal potencies of the insect tissue.

Aside from differences due to variation of material and technique the data obtained in different species of insects including Leucophaea (for reviews see Scharrer, 1941; Bodenstein, 1942; Piepho, 1943) compare well with the findings of Wigglesworth in Rhodnius. His interpretation has not remained undisputed (Pflugfelder, 1937, 1938, 1939, 1940), but his basic concept still seems to offer the most satisfactory

point of departure for an understanding of the phenomena observed, at least regarding certain orders of insects (Hemiptera, Orthoptera, Lepidoptera).

In holometabolous as well as hemimetabolous insects each developmental step may be viewed as governed by a balanced interaction between two developmental hormone systems on one side and the growing and differentiating tissue on the other. The two types of hormones collaborating in the control of development are: (1) the "growth and differentiation hormone" (GD hormone or hormones), and (2) the juvenile hormone (inhibitory hormone, corpus allatum hormone). The broader term "growth and differentiation hormone" has been proposed (Scharrer and Scharrer, 1944) for active principles of the kind previously called molting, pupation, or metamorphosis hormones in order to indicate the action they have in common (see also Bodenstein, 1943, Vogt, 1943).

A GD hormone (such as the "molting hormone" of Wigglesworth) when acting alone stimulates growth and causes or at least allows developing tissues to differentiate into imaginal ones at a rate inherent in the tissue. It brings about molts coupled with metamorphosis, but it does not cause larval (or nymphal) molts in the absence of the juvenile factor, a point which has to be kept in mind when the term molting hormone is used. In normal development the interaction between the two developmental hormones takes place for long enough a time to insure sufficient periodic steps of growth before imaginal differentiation sets in. In holometabolous insects where imaginal differentiation starts with the conclusion of the larval period (Bodenstein, 1943, Piepho, 1943), pupation and metamorphosis can be explained as caused by one or perhaps several GD factors in the absence of the juvenile hormone.

In a hemimetabolous insect such as Rhodnius and Leucophaea each consecutive nymphal molt differs from the preceding one in that it brings the insect nearer the adult stage by a small step. Thus imaginal differentiation takes place at a slow rate in all stages with the exception of the last where the more obvious changes occur. Theoretically this can be accomplished in two ways:

(1) The amount of juvenile hormone released into the circulation decreases in consecutive stages so that the hormone balance is shifted in favor of the GD hormone, whereas the responsiveness of the developing tissues does not change appreciably.

(2) The ratio of both hormones remains approximately the same in each stage (except the last), but there is an increasing readiness of the tissues to respond to the stimulus for differentiation.

It seems that both alternatives play a role, and the extent to which each prevails may vary in different groups of insects. The importance of tissue responsiveness as a factor in development can be estimated by a comparison of the effect of allatectomy in various insect species.

In all cases studied so far absence of the corpora allata results in precocious development of adult characters, but the effects appear to differ in degree. Whereas "diminutive adults" were obtained at the molt following the operation even from first instars of Rhodnius (Hemiptera; Wigglesworth, 1934), younger allatectomized nymphs of Leucophaea (fifth and sixth instars) resulted in intermediate forms (preadultoids) which required an additional molt to become adulttoids. In this respect Leucophaea resembles another Orthopteran, Dixippus, which was studied by Pflugfelder (1937, 1938, 1939). Hence in these Orthoptera allatectomy has different results when performed in earlier or later nymphal stages. These observations suggest that the young nymphal tissue of Leucophaea and Dixippus is less ready than older tissue to react to the stimulus exerted by the GD factor alone. Therefore, it may well be that in these insects the ratio of developmental hormones remains approximately the same in all stages except the last. On the other hand, in Rhodnius for instance, where nymphal tissues proved ready for adult differentiation at a very early stage, a gradually decreasing activity of juvenile hormone may take place during nymphal development. Generally it appears that the responsiveness of the tissues to developmental hormones changes with age (see also Bodenstein, 1943).

SUMMARY

Extirpation of the corpora allata was performed at various stages of development in Leucophaea maderae (Panchlorinae, Orthoptera). In eighth (last) instar nymphs the operation had no visible effect on development.

Allatectomy at stages earlier than the last caused an abbreviation of development which resulted in animals with adult-like characters ("adultoids"). In operated seventh instars the following nymphal molt was suppressed, and the animals emerged as adultoids, resembling normal adults except for their smaller size and comparatively shorter wings. Allatectomized sixth or fifth instars resulted in "preadultoid" stages which showed less adultoid differentiation and required an additional molt before becoming adultoids.

The "critical period," i.e., the time at which within a given nymphal stage an effective concentration of the corpus allatum hormone (juvenile hormone) is reached lies near the beginning of the second half of the intermolt period.

The interaction of hormonal factors and tissue responsiveness in the control of insect development is discussed.

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THE RELATIONSHIP BETWEEN CORPORA ALLATA AND REPRODUCTIVE ORGANS IN ADULT LEUCOPHAEA MADERAE (ORTHOPTERA)[1]

BERTA SCHARRER

From the Department of Anatomy, Western Reserve University
CLEVELAND, OHIO

THE INFLUENCE of the corpora allata on the activity of the reproductive organs has been studied in several insect species. Most authors agree that the corpora allata are necessary for the normal function of the ovary, but not of the testis (Wigglesworth, 1936; Thomsen, 1940, 1942; Vogt, 1940, 1942; Day, 1943; but see Bounhiol, 1939). Among the Orthoptera only two species have been studied so far. In Melanoplus differentialis Pfeiffer (1939, 1945) demonstrated that removal of the corpora allata prevents the development of the ova and the secretory activity of the oviduct. On the other hand, according to Pflugfelder (1937, 1939) egg maturation proceeds in allatectomized females of Dixippus morosus, unless the corpora allata are removed during an early nymphal stage. Because of this discrepancy the problem of the relationship between corpora allata and reproductive organs has been studied in another Orthopteran, Leucophaea maderae.

MATERIAL AND METHODS

Leucophaea maderae is an ovoviviparous insect which has been bred in the laboratory for several years under conditions briefly outlined in a preceding paper (Scharrer, 1946). The maximal adult life span observed, counted from the emergence of the imago, was 21 months for the female, 18 months for the more active male.

Allatectomy was performed as previously described (Scharrer, 1946). The reimplantation of the corpora allata was carried out in two ways: either the glands were immediately returned to the head region from where they had been removed, or, in the majority of cases, they were implanted into the abdomen. In the latter case they were taken up from saline in a fine glass capillary connected with a plunger device. In a considerable number of cases the completeness of the allatectomy was checked in serial sections through the head region of the operated animals. This material as well as the other tissues sectioned were stained according to Foot's modification of Masson's trichrome stain. Gonadectomy was performed through small transverse incisions in the respective intersegmental membranes. The gonads were pulled out carefully with a forceps, and the oviducts or vasa deferentia were severed.

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RESULTS

Extirpation and reimplantation of corpora allata

Females—In Leucophaea the development of the eggs up to the time of the hatching of the young takes place within the body of the female. The first "birth" of young occurs approximately 12 to 15 weeks after the emergence of the female imago. The reproductive cycles which follow, i.e. the intervals between successive births take similar periods of time. A maximum of seven births was recorded in a female which was sacrificed at the adult age of 21 months. The oldest (terminal) oocytes in the ovaries measure about 1 to 1.2 mm. in length at the beginning of a reproductive cycle, and they reach a length of 5 mm. before "ovulation" occurs. This phase of growth during which yolk is deposited requires about four to five weeks. In histological preparations of developing eggs yolk can be demonstrated as soon as the first increase in size is noted (fig. 1). Further development takes place in the uterus where the embryos, up to 40 in number, are ensheathed and packed together by a common membrane.

For the normal course of egg development in Leucophaea the presence of the corpora allata is necessary. This was demonstrated by the following experiments.

In 79 adult females total extirpation of the corpora allata was performed one to seven days after emergence. An additional group of four females had been allatectomized in the last nymphal stage. Unless damage to other structures such as the esophagus or the recurrent nerve (Scharrer, 1945) occurs, the animals make a quick recovery after the operation and show no externally visible difference from normal controls. They were kept under observation together with adult males for periods up to 13 months after allatectomy. The mating behavior seemed unchanged after allatectomy.

In none of these females did the eggs develop appreciably beyond the stage typical of those of the freshly emerged imago, i.e., their length did not exceed 1.4 mm., and yolk deposition did not take place (fig. 2). Likewise, the accessory sex glands showed little or no sign of secretion (fig. 5) and, after several weeks, when normal control glands were at the height of their activity (fig. 6), resembled those of a newly emerged imago. Fourteen female adultoids (Scharrer, 1946, p. 37) resulting from nymphs that had been allatectomized in the seventh stage also showed no development of the eggs and no secretion of the accessory sex glands.

In two additional "allatectomized" adults which gave birth to young, subsequent histological examination showed that the extirpation had been incomplete. One of the remnants of corpus allatum tissue had proliferated. This finding demonstrates that enough glandular tissue may regenerate from a fraction of the original glands left behind at the operation to make egg development possible. Additional information concerning this point was gained by a series of experiments in

which unilateral allatectomy was performed in ten animals shortly after emergence. In such females egg development proceeded normally. No significant delay was noted in the births of the young as compared with normal control animals.

In three animals allatectomized shortly after they had given birth to young no further egg development occurred. Thus the corpora allata appear to be necessary not only for the first but also for successive reproductive cycles in the female.

In order to demonstrate that the inability of the ovaries to develop normally after allatectomy was not merely due to trauma, sham operations were performed in 17 females shortly after they emerged. The details in these operations were like those in allatectomy except that the corpora allata were not removed. Egg development was not impaired in these females. However, failure of the eggs of Leucophaea to undergo development has been observed also under conditions other than those of allatectomy. Removal of the brain or starvation (fig. 3; Scharrer, 1943) suppressed the ovaries, at least in a certain percentage of experimental animals. Cutting of the recurrent nerve or extirpation of the frontal ganglion, both resulting in tumor formation (Scharrer, 1945) had similar results. In order to prove that these effects on the ovary are less specific than that obtaining after allatectomy, it was necessary to reimplant corpora allata into females that had been allatectomized shortly after emergence.

Among 67 of these animals with allatum grafts 61 showed egg development and secretory activity of the accessory sex glands (figs. 4 and 7). Some of them kept for over six months gave birth twice, which observation suggests that in these cases the implants remained functional for a long time. Fifteen batches of young that had developed under the influence of the implanted corpora allata were raised separately. In appearance, behavior, and further course of development they differed in no way from young of normal females. In six females the implants probably did not take. It was not possible to recover these implants and to study them histologically.

All these experiments demonstrate that in Leucophaea the corpora allata are necessary for egg development. The question arises whether their presence in the mother is required throughout the embryonic

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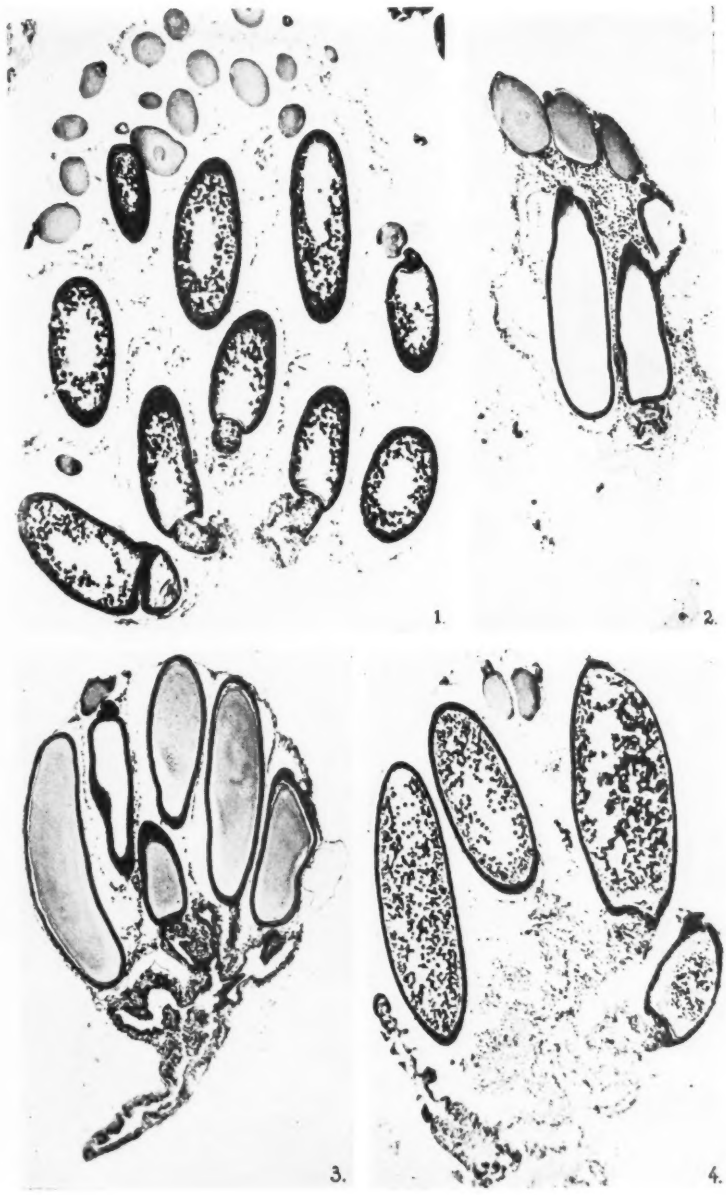
FIG. 1. Ovary of normal female of Leucophaea maderae fixed shortly after beginning of reproductive cycle. Largest oocytes (length measured before fixation 1.3 mm.) contain yolk. Zenker-formol, paraffin, 7μ, Masson. ×45.

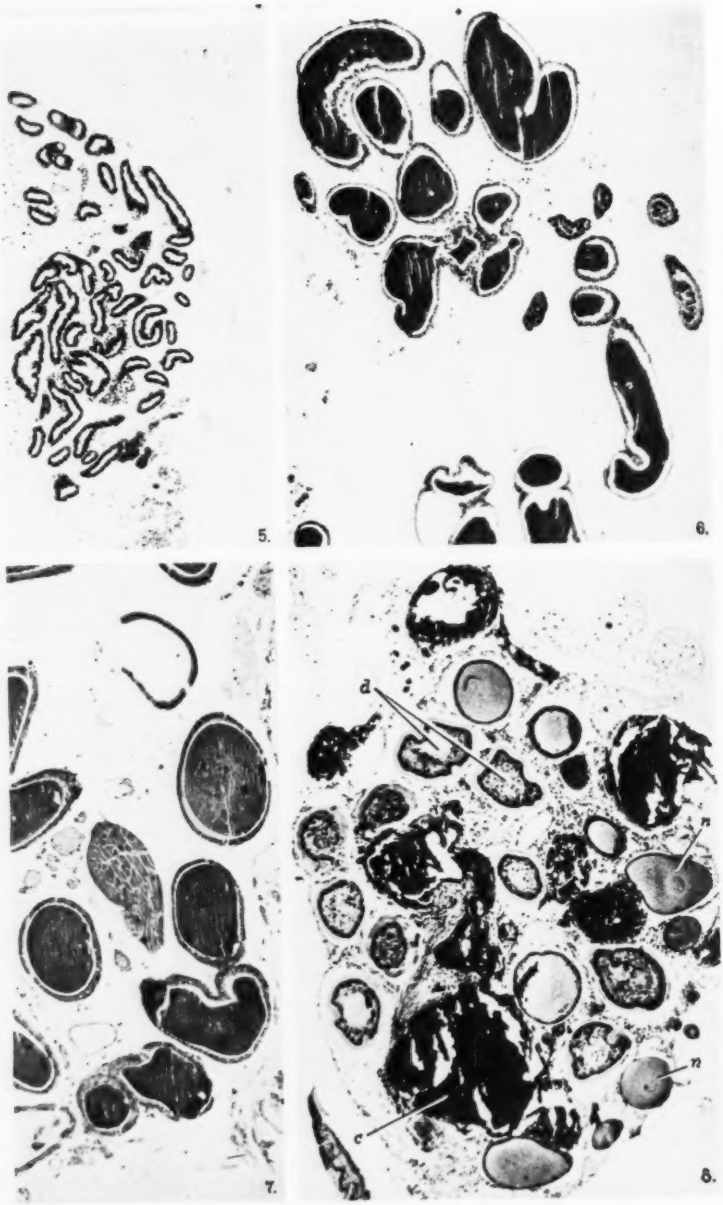
FIG. 2. Ovary of animal allatectomized shortly after emergence and killed six weeks later. Largest oocytes, 1.1 mm. in length, resemble those of freshly emerged adult. Note absence of yolk. Zenker-formol, paraffin, 7μ, Masson. ×45.

FIG. 3. Ovary of female starved for 41 days after emergence. Eggs failed to grow and no yolk was deposited. Zenker-formol, paraffin, 7μ, Masson. ×45.

FIG. 4. Ovary of female whose corpora allata were reimplanted after allatectomy. Fixation about two weeks after beginning of reproductive cycle. Largest oocytes resemble normal ones of comparable age. Zenker-formol, paraffin, 7μ, Masson. ×45.



Figs. 1–4. See opposite page for description.



Figs. 5–8. See opposite page for description.

phase or whether development may proceed autonomously after an initial stimulus from the corpora allata has been received. Information on this question is furnished by the following experiments. Forty

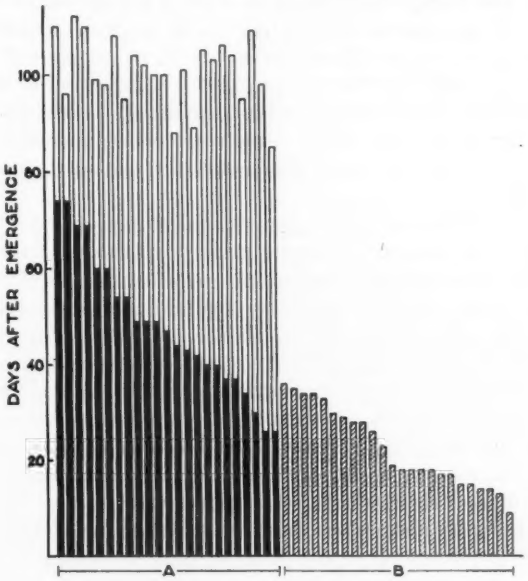


FIG. 9. Diagram indicating effect on egg development of allatectomy at various intervals after emergence. A. Group of 23 females allatectomized 26 to 74 days after emergence; all gave birth to normal young. Black: intervals between emergence and allatectomy; white: intervals between allatectomy and birth. B. Group of 24 females allatectomized nine to 36 days after emergence; from the time of allatectomy egg development ceased in all these females.

seven females were allatectomized at intervals ranging from nine to 74 days after emergence. The results obtained from these experiments (fig. 9) can be arranged in two groups:

A. Twenty three animals that subsequently gave birth to young

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FIG. 5. Accessory sex glands of female allatectomized shortly after emergence and killed six weeks later (same animal as in figure 2). Glands resemble those of freshly emerged normal female which also do not secrete. Compare with figures 6 and 7. Zenker-formol, paraffin, 7μ, Masson. $\times$45.

FIG. 6. Accessory sex glands of normal control female in state of active secretion. Zenker-formol, paraffin, 7μ, Masson. $\times$45.

FIG. 7. Actively secreting accessory sex glands of female whose corpora allata were reimplanted after allatectomy. Fixation after approximately first third of reproductive cycle. Zenker-formol, paraffin, 7μ, Masson. $\times$45.

FIG. 8. Transverse section through ovary fixed ten months after allatectomy. n, normal oocytes; d, degenerating oocytes with proliferating follicular epithelium; c, cysts containing brown debris. Zenker-formol, paraffin, 7μ, Masson. $\times$45.

had been operated on 26 to 74 days following their emergence. In these cases embryonic development was not slowed down as compared with normal controls. The average duration of the reproductive cycles was 100.7 days (14½ weeks), with a minimum of 85 days (12 weeks) and a maximum of 110 days (15½ weeks). In this group the presence of the corpora allata for a period of at least 26 days after emergence was sufficient to safeguard the completion of egg development. In none of the females of this group did further egg development take place after the first birth. This was to be expected, because allatectomy after the first birth also prevented the occurrence of further births (p. 48).

B. In those females whose eggs did not complete development the interval between emergence and allatectomy ranged from nine to 36 days. When these animals were sacrificed after two to ten months the ovaries frequently showed signs of degeneration similar to those described by Wigglesworth (1936), Pfeiffer (1939), Pflugfelder (1940), and Day (1943). Figure 8 shows the ovary of a female which had been allatectomized 33 days after emergence and was allowed to survive for ten months following the operation; no young were born throughout this period. The majority of oocytes are in the process of degeneration, as indicated by the irregular outlines and the proliferation of the follicular cells which results in the disappearance of the oocytes. Large brown cysts finally take their place. Similar signs of regression may occasionally be observed in normal old females which have not produced young for a long period (see also Day, 1943).

In summary it can be stated that in all females operated on at an interval of more than 36 days after emergence egg development continued in the absence of the corpora allata. The eggs of all females operated on less than 26 days after emergence did not complete their development. Operations carried out in the period between 26 and 36 days after emergence produced animals with or without egg development.

The conclusion to be drawn from these results is that the corpora allata are necessary for about four to five weeks, i.e. roughly during the first third of the total period required for egg development. During the subsequent two thirds of this period the development up to the time of hatching may proceed in the absence of the corpora allata. The time during which the eggs are under the influence of the corpora allata corresponds to the period of growth and yolk deposition. Independence from the corpora allata appears to be reached after ovulation when differentiation sets in.

Males.—The influence of the corpora allata on the reproductive organs of male Leucophaea was studied in 100 animals with total and 28 animals with unilateral allatectomy. In the majority of these cases the operations had been performed one to seven days after the emergence of the adults; some were operated on considerably later (up to

11 months after emergence), others earlier (during the last nymphal stage). In the male the operation did not seem to impair sexual activity. Nine of the allatectomized males, when mated with normal virgin females, were capable of fertilizing the eggs. Histologically, testes and accessory sex glands of allatectomized animals did not appear significantly different from those of unoperated animals. Thus extirpation of the corpora allata in male Leucophaea appears to have no influence on the maintenance of reproductive activity which result is in contrast to that in the female.

Castration

Additional information on the interrelationship between reproductive organs and corpora allata was sought by castration experiments. Forty one males of Leucophaea had both testes removed either in late nymphal stages or shortly after emergence. Similarly 28 adult females were ovariectomized six to 18 days after emergence. The animals were killed at intervals ranging from one to 14 months after the operation. Histologically the corpora allata of castrated animals showed no significant hypertrophy or changes in staining properties and cellular appearance.

In castrated females the accessory sex glands were actively secreting. This demonstrates that the secretory activity of these glands is governed by the corpora allata directly, and not by way of the gonads. In some older castrates excessive amounts of the secreted materials accumulated, obviously because it was not utilized in the absence of the ovaries.

DISCUSSION

Extirpation and reimplantation experiments in Leucophaea maderae demonstrate conclusively that the development of the eggs, for a certain period of time, proceeds under the hormonal control of the corpora allata. The data presented here support the findings of Pfeiffer (1939) in Melanoplus against the result of Pflugfelder (1937, 1939, 1940) in Dixippus. They are also in agreement with the observations reported in Hemiptera (Wigglesworth, 1936) and in Diptera (Thomsen, 1940, 1942; Vogt, 1940; Day, 1943). No effect of the corpora allata on egg development has yet been demonstrated in Lepidoptera (Bounhiol, 1939).

Thus a relationship between the corpora allata and the ovaries seems to exist in the majority of insect species. However, the nature of this hormonal action is still under dispute. It has been demonstrated conclusively by Pfeiffer (1945) that the corpora allata are the source of a hormone which controls the metabolism of fat and the mobilization of some non-fatty material (metabolic hormone). This leads to the question whether the effect on the female reproductive organs is brought about by this metabolic hormone, or whether there exists in addition a specific "gonadotropic" hormone.

There is to date no definite proof for the presence of a gonadotropic hormone in insects. Its existence need not be postulated as long as the experimental data at hand can be explained on the basis of Pfeiffer's findings. In Melanoplus the effect of allatectomy does not manifest itself before yolk deposition begins. A similar situation seems to exist in Rhodnius (Wigglesworth, 1936) and in Calliphora (Thomsen, 1942). The data in Leucophaea reported here are in line with these findings. In this species egg development was shown to depend on the corpora allata up to the time of ovulation, i.e. throughout the period of growth and yolk deposition. Hence in all species studied so far the inability of the eggs to continue development after allatectomy can well be attributed to the absence of the metabolic hormone.

The secretory activity of the accessory sex glands in Leucophaea appears to have its counterpart in that of the epithelial lining of the oviducts in Melanoplus. Both structures depend for their normal function on the corpora allata. It is not known whether the hormone acting on these glands is the same as that acting on the ovaries. Its influence on the accessory glands (or oviducts) is direct, and not by way of the gonads. This is proved by the fact that in ovariectomized females of Melanoplus as well as Leucophaea the secretory activity of these glands is maintained.

In Diptera Thomsen (1942; Calliphora) demonstrated a certain correlation between corpora allata and accessory glands of the reproductive system in both sexes. On the other hand, Day (1943; Lucilia, Sarcophaga) found no influence of allatectomy on these glands in either females or males. The male glands of Hemiptera (Rhodnius) need for their proper functioning the presence of the head, i.e., the corpus allatum (Wigglesworth, 1936). As far as the testis is concerned, no relationship with the corpora allata has been demonstrated. Attempts to disturb the course of spermatogenesis by the extirpation of the corpora allata had negative results in Rhodnius (Wigglesworth, 1936) and Calliphora (Thomsen, 1942) as well as in Leucophaea.

As regards castration no influence on the corpora allata was observed in males of Lucilia (Day, 1943) or Leucophaea. The data concerning the effect of ovariectomy on the corpora allata are divergent. This operation leads to hypertrophy of the corpora allata in the Orthopteran Melanoplus (Pfeiffer, 1940) and in some Diptera (Calliphora, Thomsen, 1940, 1942; Lucilia, Day, 1943; see also Vogt, 1942), but not in others (Sarcophaga, Day, 1943). According to Day, however, there are indications that the corpora allata of ovariectomized Sarcophaga are "physiologically altered," even though their histological appearance shows no significant change. In Leucophaea a similar interpretation of the negative histological result may be possible, but at present cannot be based on experimental data.

SUMMARY

In Leucophaea maderae (Orthoptera) the presence of the corpora allata is necessary for the development of the eggs.

In females allatectomized shortly after emergence the eggs did not develop appreciably (maximum length of oocytes 1.4 mm.) beyond the stage in which they are found in the young imago. The corpora allata are needed not only for the first but also for successive reproductive cycles. The accessory sex glands in these operated females showed little or no sign of secretion in contrast to normal control glands.

No delay or other visible disturbance of egg development was observed after unilateral extirpation of the corpora allata.

Reimplantation of corpora allata into allatectomized females caused the eggs and the nymphs hatching from them to develop as normally as those of unoperated animals.

In a series of experiments in which the time of allatectomy was varied it was demonstrated that the corpora allata are necessary throughout the period of growth and yolk deposition which constitutes about the first third of the total period required for the development of the eggs.

Allatectomized males when mated with normal virgin females were capable of fertilizing the eggs. The corpora allata are apparently not necessary for the reproductive activity of the male which result differs from the situation in the female.

After castration the corpora allata of females and males showed no significant change in size or histological appearance. The accessory sex glands of ovariectomized females were actively secreting.

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NOTES AND COMMENTS

METHODS FOR CONCENTRATION OF RELAXIN FROM BLOOD SERUM AND URINE[1]

A SUBSTANCE, relaxin, capable of relaxing the symphysis pubis of the guinea pig has been discovered in pregnancy serum of several species of animals (Hisaw, 1929) and man (Pomerenke, 1934). The greatest concentration, 10–12 units per ml. serum and 0.5 units per ml. urine apparently occurs in the rabbit (Marder and Money, 1944). In other forms, the concentration of relaxin is evidently much less and in some cases, it has not been possible up to this time to assay for it because the test animals cannot usually tolerate more than 5 ml. serum or 10 ml. urine. It is the purpose of this note to describe methods whereby the relaxating substance can be concentrated so that 5–10 times greater amounts of serum and 10–20 times greater amounts of urine can be given to the test animal without toxicity.

Blood Serum. One volume of cold ethyl alcohol is added slowly to one volume of serum. After standing 15 to 30 minutes, the mixture is centrifuged. The precipitate is dissolved in one volume of saline and mixed with one volume of alcohol as before. After again centrifuging, the precipitate is discarded. The combined clear supernatant fluid is poured into four volumes of cold acetone. A white precipitate forms which is collected by centrifuging and suspended in $\frac{1}{5}$ volume of water or saline. This concentrate is now ready for assay as described by Abramowitz et al., 1944. The equivalent of 25 to 50 ml. of serum can be injected into guinea pigs without toxicity, probably because the concentrate contains only $\frac{1}{3}$ of the total solids of the serum.

As applied to carefully assayed pregnant rabbit serum, this method has yielded complete recovery in the concentrate of all the activity of the original serum. No activity could be detected in the 50% alcohol precipitate or in the alcohol acetone fraction, the sum of which contain the remaining $\frac{2}{3}$ of the solids of the serum before fractionation. A further test of the efficacy of this method was made by adding one unit of relaxin from pregnant rabbit serum (0.1 ml.) per 10 ml. of male rabbit serum which contains no relaxin. This mixture (containing only 1/100 the relaxin concentration during late pregnancy in the rabbit) was concentrated by the above method with complete recovery of the added relaxin. Methods previously suggested, i.e., the removal of the pseudoglobulin fraction of serum by salting out methods (Hisaw, 1929) or by acid extraction of serum (Abramson et al., 1937) are not entirely suitable for this purpose since losses of 50% and 30% respectively of the original activity occurred even under the most favorable conditions.

Urine. Twenty-four hour specimens of urine are collected under toluol and dialyzed against running tap water for 12 hours, and against distilled water for 3 hours in a rocking dialyzer. The urine is reduced to 1/20 its

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[1] Aided by a grant from the Milton Fund to Professor F. L. Hisaw.

original volume by evaporation in a current of warm air and then poured into four volumes of cold acetone.[2] A few drops of saturated NaCl are added to facilitate precipitation. The precipitate is collected by centrifuging and suspended in water or saline so that each ml. of suspension is equivalent to 100 ml. of original urine. This suspension is easily tolerated by the test animals.

The efficacy of this method was tested by adding known amounts of ovarian relaxin to boiled and unboiled human male urine, and assaying aliquots during the procedure. An overall loss of about 10% occurred, during dialysis. Of over a dozen different methods tried to precipitate relaxin from urine by various agents, this method proved the only one in which the toxicity of the concentrate was lowest and the total recovery (90%) greatest. The content of a relaxating substance[3] in the urine of pregnant women was found by this method to be 10–50 units per 24 hour sample depending on the length of gestation.

Summary. Methods are described for concentrating relaxin from blood serum and urine. Serum can be concentrated without measurable loss and toxicity so reduced that 5–10 times greater amounts of serum can be assayed. Urine can be concentrated with a 10% loss in relaxin content, and toxicity so reduced that 10–20 times greater amounts can be tested.

ALEXANDER ALBERT[4] AND W. L. MONEY

From the Biological Laboratories,
Harvard University,
Cambridge, Massachusetts

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[2] Reduction of volume by evaporation was instituted to avoid the use of large volumes of acetone.

[3] It is not implied that relaxating substances from the three different sources (ovarian, serum, urine) are necessarily identical, since evidence in this direction is as yet insufficient.

[4] Present Address: Thyroid Clinic, Massachusetts General Hospital, Boston.

A NOTE ON THE MODE OF THIOURACIL ACTION

In considering the thyroid effect of thiourea and other goitrogenic chemical compounds, including thiouracil and the sulfonamides, the following mechanisms have been proposed by MacKenzie and MacKenzie (1943); a) Retention of thyroxin by the thyroid gland, b) Elevation of tissue requirements for thyroxin (e.g. through the inhibition of some enzyme system), c) Destruction or inactivation of the circulating hormone, d) Depression of the rate of thyroxin formation. Recent publications (Larson, Keating, Peacock and Rawson, 1945; Reineke, Mixner and Turner, 1945; Leathem, 1945) support the postulation of Astwood, Sullivan, Bissell and Tyslowitz (1943) that the action is directly upon the thyroid gland so that there is interference with the production of the thyroid hormone. Inhibition in the secretion of the hormone permits an increase of thyrotropic hormone secretion by the pituitary and a compensatory hypertrophy of the thyroid gland. The following experiments demonstrate that thiouracil does not inactivate and is not antagonistic to preformed and circulating thyroxin.

METHODS

Hunt (1905, 1907, 1907a, 1911, 1923, 1925), Hunt and Seidell (1908, 1910, 1911), Carlson and Woelfel (1910) and others using thyroxin, thyroglobulin, dessicated thyroid gland and other thyroid preparations consistently noted that these substances protected white mice against acetonitril poisoning. The modification of this technic by Fellinger and Hochstädt (1935) by which the protection afforded by thyroxin is removed by antithyroidal substances has been applied recently to blood extractives by Hochstädt and Malkiel (1944). This modification of the Reid Hunt method was carried out in these experiments.

Young, female, white mice weighing between 20–25 gm. were used. During the course of the experiment they were kept on the usual routine animal diet. Group I consisted of 12 animals receiving 2 cc. of an aqueous 0.1% thiouracil solution injected intraperitoneally daily for six days; group II, 14 animals receiving similarly 1 cc. of an aqueous 0.2% sulfaguanidine solution. Twelve milligrams of the respective drug were received by each mouse during this period. On the last three days these animals in groups I and II received a daily subcutaneous injection of 0.1 cc. of a 5% solution of crystalline thyroxin[1] (0.5 mg. daily) in dilute alkali. Group III was a control of 12 mice receiving neither thiouracil nor sulfaguanidine but only thyroxin for three days. Group IV also was a control of 13 mice receiving thiouracil for six days but with no concomitant thyroxin. Four mice of this group were sacrificed and their thyroid glands were examined for any microscopical histopathology. The M.L.D. of acetonitril for white mice was determined as approximately 0.6 mg. per gram of body weight, using a 10%

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[1] Crystalline Thyroxin Squibb was generously supplied by Dr. H. Sidney Newcomer of E. R. Squibb and Sons.

aqueous solution. Eight hours after the last thyroxin injection, groups I, II and III received twice the M.L.D. of acetonitril subcutaneously. Group IV received one M.L.D. subcutaneously. The animals were then kept under observation for 24 hours.

RESULTS

All the animals became acutely ill within five minutes after the acetonitril injection. There were four deaths in group IV after 24 hours. All the mice of group III recovered. There were no deaths in experimental groups I and II; all recovered fully. The thyroid glands of the normal controls microscopically showed a definite hyperplastic response. The acinar epithelium was uniformly cuboidal and the colloid within the acinae showed beginning vacuolization. The supporting stroma was markedly edematous and occasionally lymphocytes were found therein. This response indicated that the treatment with thiouracil was carried out in a physiologically effective manner.

SUMMARY

It is possible to demonstrate an antithyroidal effect using a modified Hunt technic. This procedure was applied to the goitrogenic chemicals, thiouracil and sulfaguanidine. Neither of these drugs showed any antagonistic effect to free circulatory preformed thyroxin. Therefore it can be established that the goitrogenic drugs do not act by destruction or inactivation of thyroid hormone.

SAUL MALKIEL

From the Departments of Pathology
Yale University School of Medicine
New Haven, Connecticut
and Boston University School of Medicine
Boston, Massachusetts

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NEW BOOK

PHYSICAL CHEMISTRY OF CELLS AND TISSUES. By Rudolf Höber, with the collaboration of David I. Hitchcock, J. B. Bateman, David R. Goddard, and Wallace O. Fenn. The Blakiston Company. $9.00.

This book is based on the premise that cellular structure and function should be explained in terms of chemistry and physics. Like D. A. W. Thompson's "Growth and Form," "Physical Chemistry of Cells and Tissues" should serve to accelerate the trend of biological thinking away from predominantly descriptive and vitalistic concepts into mechanistic and fundamental channels. It emphasizes the fact that cells can be regarded as aggregations of ions and molecules, that cell structure must be explained in terms of the localization and arrangement of these ions and molecules and cell function as manifestations of their interactions and the forces they induce. The book suggests to the reviewer that the biological sciences might benefit considerably if they succeeded in attracting a greater number of workers well trained in advanced mathematics, physics, and chemistry.

The book is divided into eight sections, of which four are written by Dr. Höber. The first section, on "Selected Principles of Physical Chemistry" by Hitchcock, and the sixth, by Goddard, entitled "The Respiration of Cells and Tissues" provide brief explanations, definitions and equations dealing with the basic concepts and principles in these respective fields. Bateman's section on large molecules draws intriguing analogies between biological structures and certain plastics and crystals. The section by Fenn on "Contractility," dealing primarily with muscle, seemed to this reviewer to be the best in the book. It offers an integration of the physiology and biochemistry of muscle action with the molecular architecture based on myosin. Höber's sections deal primarily with diffusion and interchange across cell membranes, and reflect his strong tendency to think of biological systems in terms of models. His is the dilemma of all physical chemists dealing with biological systems: confronted as they are by configurations too complex and obscure for ready analysis, they resort to simple models which lend themselves to the methods of physical chemistry. They may perhaps be forgiven if at times they appear to confuse the model with the original in their own minds, for their approach is a useful one from which biology has benefited.

One wishes for a discussion of the placenta in this book, as there are no tissues involved in permeability phenomena more challenging to the investigator. It is noteworthy that in the discussions of fluid and solute absorption by cells no mention is made of the process called "pinocytosis" by Warren H. Lewis, and portrayed so provocatively in his excellent motion pictures of living cells in tissue cultures. Such methods of fluid transfer involving actual visible motion of the cell protoplasm may well be widespread and important in many tissues. Perhaps the book gives insufficient attention to the proteins themselves, and it is to be regretted that there is not included a discussion of the chemistry and energetics of synthetic processes.

"Physical Chemistry of Cells and Tissues" is a most stimulating and informative volume. It presents a great wealth of references, including lists of pertinent textbooks and monographs. Its effort to interpret cellular phenomena in terms of ions and molecules and their arrangement and interactions outlines the basis and the direction of future advances in fundamental biology. The book should be well-received and usefully employed in all laboratories embracing this field.

H. Stanley Bennett

Department of Biology,
Massachusetts Institute of Technology,
Cambridge, Massachusetts

BOOKS RECEIVED

Constitutional Medicine and Endocrinology. Edited by E. Pulay, M.D.A.P. A. W. Cawadias, O.B.E., M.D., F.R.C.P. P. Lansel, M.D. Frederick Muller Ltd. Vol. I. London.

Die Spezielle Pathologie und die Verletzungen der Mundgebilde. Für Arzte, Zahnarzte und Studierende der Medizin und der Zahnheilkunde. Prof. Dr. Med. A. Fonio. Professor für Chirurgie an der Universitit Bern. Medizinischer Verlag Hans Huber, Bern.

Estudios de Histofisiologia e Histofisiopathologia Tiroidea. Por Washington Buno Profesor Titular de Histologia y Embiologia de la Facultad de Medicina. Jefe de la Seccion Histologia del Instituto de Endocrinologia Ano 1943 Montevideo Editor El Ateneo Florida 340 Cordoba 2099 Buenos Aires. 200 pp.

Secretory Mechanism of the Digestive Glands. By B. P. Babkin, M.D., D.Sc., LL.D., F.R.S.C. Research Professor of Physiology, McGill University, Montreal, Canada; Formerly Professor of Physiology in the University of Odessa, Russia, and in Dalhousie University, Halifax, Nova Scotia, Canada. Published by Paul B. Hoeber, Inc. Medical Book Department of Harper & Brothers, New York. London. pp. 900, 220 Ill., Price $12.75.

Temas Endocrinos, Hipofisis, Suprarrenal, Tiroides, Endometrio. By Pablo E. Borras. Editor El Ateneo, Buenos Aires.

The South African Frog (Xenopus Laevis) in Pregnancy Diagnosis. Abner I. Weisman and Christopher W. Coates. Research Bulletin, New York. Biologic Research Foundation, New York. 1944. XIV+134 pp.

Structure and Function of the Human Body. By Ralph N. Baillif, Ph.D. Assistant Professor of Anatomy, Louisiana State University School of Medicine, New Orleans, and Donald L. Kimmel, Ph.D. Associate Professor of Anatomy, Temple University School of Medicine, Philadelphia. Published by J. B. Lippincott Company, London, Philadelphia, Montreal. 328 pp., 164 Illustrations, Price $3.00.

ANNOUNCEMENT

THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

Office of the Secretary-Treasurer
1200 North Walker Street
Oklahoma City, Oklahoma
February 7, 1946

Announcement of the Annual Meeting

The Twenty-Eighth Annual Meeting of The Association for the Study of Internal Secretions will be held in San Francisco, California, Friday and Saturday, June 28th and 29th, 1946 at the St. Francis Hotel. Due to crowded hotel and transportation facilities you are urged to make these reservations immediately. Hotel Reservations should be made through Dr. M. Laurence Montgomery, Chairman Hotel Committee, Room 200, 61 Grove St., San Francisco 2, California.

The Chairman of the Local Committee is Dr. Hans Lisser, Fitzhugh Bldg., San Francisco 8, California.

Presentation of Papers

1. The title of the paper and four copies of a comprehensive abstract should reach the President, Dr. Carl Moore, Dept. of Zoology, University of Chicago, Chicago 37, Illinois, not later than April 1st, 1946.

Abstract submitted should be in proper form for printing in the program. Not more than the first 200 words can be included in the printed abstract.

2. Non-members who wish to present papers must have their titles and abstracts introduced by members.

3. The abstracts of papers will be considered by the Program Committee and the final program announced about May 1st.

4. Papers will be limited to ten minutes for presentation. A ten-minute presentation is designed for condensed discussion of new investigations, either in laboratory or clinical phases of endocrinology. Previous publication or presentation before other societies of national membership may be cause for omitting a paper from the program.

5. Papers presented at the Annual Meeting may be submitted for publication to the Editor of Endocrinology or *The Journal of Clinical Endocrinology*. Such submission is invited. Acceptance of a paper for the program does not necessarily mean its acceptance for publication.

You are invited to bring this announcement to the attention of your colleagues and associates who have endocrine research in progress.

Nomination of Officers

The By-Laws of the Association provide that nominations for all elective offices shall be made by a Nominating Committee and forwarded to the

Secretary at least sixty days before the annual meeting. The Nominating Committee for the current year, appointed by the President and accepted by the Council, is as follows:

Dr. Fred C. Koch (Chairman) 1425 W. 42nd St., Chicago, Illinois
Dr. George Thorn, Peter Bent Brigham Hospital, Boston, Mass.
Dr. Willard Allen, 630 South Kingshighway Blvd., St. Louis, Missouri

The By-Laws also provide (Article V, Section 2) that "Any member of the Association may submit nominations to the Nominating Committee for its consideration." Nominations may be sent to the Chairman, Dr. Fred C. Koch, before March 15, 1946.

The terms of the following officers expire at the time of the 1946 Annual Meeting in San Francisco.

President-Elect	Fuller Albright
Vice-President	Willard O. Thompson
Secretary-Treasurer	Henry H. Turner

Council Members

E. L. Sevringhaus R. G. Hoskins Earl T. Engle

THE SQUIBB AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner, in 1941 to Dr. Philip E. Smith, in 1942 to Dr. Fred C. Koch, in 1944 to Dr. E. A. Doisy, in 1945 to Dr. E. C. Kendall, no Award was given in 1943. A special Committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. Dr. E. B. Astwood received the award in 1944, Dr. Jane A. Russell in 1945. The work cited may be either in the field of pre-clinical or clinical endocrinology. The Award is for $1,200.00. If the recipient should choose to use the Award toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00. The option is left entirely to the recipient.

Each member has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of the nominee's most important publications, and reprints if possible. Five copies should be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma not later than March 15, 1946.

CARL R. MOORE, *President*
HENRY H. TURNER, *Secretary*